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Jeho Shin

*Louisiana State University and Agricultural and Mechanical College, [jshin2@lsu.edu](mailto:jshin2@lsu.edu)*

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**THE EFFECT OF AGE AND SEX ON THE GROWTH PATTERNS  
OF BOVINE CELL LINES**

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

in

The Interdepartmental Program in Animal and Dairy Sciences

by  
Jeho Shin  
B.S., Konkuk University, 1991  
M.S., Konkuk University, 1994  
May, 2005

## **DEDICATION**

This thesis is dedicated to my mother, Kyung-soon, who prayed for me and my father, now in heaven, are two persons whom I thank most of all. I also want to dedicate this work to my wife, Jung-rim, and son, Tae-soo, who have shared joy and sorrow with me.

## **ACKNOWLEDGMENTS**

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## ABBREVIATIONS USED IN THESIS

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B-bulls	ICM-inner cell mass
C-cows	IVF- <i>in vitro</i> fertilization
CV-coefficient of variation	MC-male calves
DMSO-dimethyl sulfoxide	Mdc-muscle derived cell
DMEM-dulbecco's modified eagle medium	MGT-mean cell generation time
EDF-epidermal cell derived factor	NT-nuclear transfer
EGF-epidermal growth factor	PBS-phosphate-buffered saline
ES-embryonic stem	PRB-retinoblastoma protein
FACS-fluorescence activated cell sorter	SPCC-stationary phase viable cell count
FBS-fetal bovine serum	TE-trophectoderm
FC-female calves	TERT-telomere reverse transcriptase
FGF-fibroblast growth factor	TIG1-human fetal lung fibroblast
HBSS-Hanks' balanced salts solution	TRF-telomere repeat binding factor
IGF-insulin-like growth factor	

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## ABSTRACT

The influence of donor animal sex or age on *in vitro* bovine cell culture was evaluated to provide foundation information for the selection of donor tissue for nuclear transfer. Skin biopsies were taken from each of sixteen individuals including four bulls (B), four cows (C), four male calves (MC), four female calves (FC). At passage 2, cells from *in vitro* culture of cell lines were influenced not by gender but by age in the mean cell generation time (MGT). When evaluating familial lineage, comparison between related and unrelated groups showed that most comparisons do not show significant differences in lag time, stationary phase viable cell counts (SPCC) and MGT. In each cell line, there was high cell viability throughout the growth curves, indicating stable cell maintenance and proper cell harvest was conducted in this study. At passage 4, MGT of each cell line was not influenced by age but by sex at passage 2, however, at a later cell passage (by passage 4), the MGT of each cell line was not affected by either sex or age of the donor. By passage 4, the MGT of each cell line was not affected by either sex or age. As passages continued, the extrinsic environmental factors likely influenced the MGT. Cell cycle analysis at passage 4 on day 0 of this study showed that >90% of cells were in G<sub>0</sub>/G<sub>1</sub> portion in each cell line of all groups. Cell lines from younger donors were more frequently at higher G<sub>0</sub>/G<sub>1</sub> percentages, or synchronized than those derived from older donors. Thus, age of donor animal could be a factor in selecting cell line for NT, especially when G<sub>0</sub>/G<sub>1</sub> nuclei are intended for use. Male groups (B and MC) showed higher stationary phase viable cell counts than female groups (C and FC). Most comparisons showed no significant differences in lag time, SPCC and MGT between related and unrelated familial lineage groups. Each cell line showed constant viability (94.36 to 97.98%) at passage 4 throughout the cell growth curves.

## CHAPTER I

### INTRODUCTION

The first mammal cloned from an adult somatic cell was born in 1997, a sheep named “Dolly” (Wilmut *et al.*, 1997). The sheep was created from the transfer of a nucleus from a differentiated somatic cell into an enucleated oocyte (Wilmut *et al.*, 1997). Though the somatic cell nuclear transfer (NT) technique has been used with various mammalian species, and has led to viable offspring (Cibelli *et al.*, 1998; Wakayama *et al.*, 1998; Baguisi *et al.*, 1999; Betthausen *et al.*, 2000; Polejaeva *et al.*, 2000; Shin *et al.*, 2002; Woods *et al.*, 2003; Galli *et al.*, 2003; Zhou *et al.*, 2003), the overall efficiency of NT is less than 1% of reconstructed embryos (Han *et al.*, 2003).

To overcome this low NT efficiency, there have been two primary approaches taken by researchers. The first approach was to investigate embryo development following NT. In attempts to increase embryonic development following NT, tetraploid complementation (Nagy *et al.*, 1990; Nagy *et al.*, 1993; Guillemot *et al.*, 1994; Duncan *et al.*, 1997; Iwasaki *et al.*, 2000; Misra *et al.*, 2001; Eggan *et al.*, 2002) or inner cell mass (ICM) transplantation (Polzin *et al.*, 1987; Anderson, 1988; MacLaren *et al.*, 1992; Lasley *et al.*, 1994; Rorie *et al.*, 1994) have been attempted.

The second main approach to increasing NT efficiency is the selection of criteria for the donor cell line. The cell cycle phase of donor nuclei is important to NT success and to embryo reconstruction (Wang, 1991; Collas *et al.*, 1992; Mosca *et al.*, 1992; Levenson and Hamlin, 1993; Kitagawa *et al.*, 1994; Di Matteo *et al.*, 1995; Prather, 1996; Campbell *et al.*, 1996; Wilmut *et al.*, 1997; Cooper, 1998; Cibelli, 1998; Wells *et al.*, 1999; Cooper, 2003; Kues *et al.*, 2000). In the first production of a mammalian offspring from adult somatic cells, most of the donor cells were arrested in a quiescent stage ( $G_0$ ) by serum starvation (Wilmut *et al.*, 1997). Though some somatic cell cloning has been achieved with donor cells in  $G_2/M$  phase (Cibelli *et al.*, 1998; Ono *et al.*, 2001), better efficiency has been obtained with the use of nuclei in  $G_0/G_1$  phase (Campbell *et al.*, 1996; Collas *et al.*, 1992; Prather, 1996).

The effects of sera on cell growth have been investigated, however, results vary in those studies where animal age is a parameter. In particular, results on cell growth are inconsistent when the serum was derived from animals of differently aged animals (Carrel and Ebeling, 1921; Baker and Carrel, 1925; Parker, 1931; Kondo and Nomaguchi, 1985; Kondo *et al.*, 1988a, 1988b). Sera from old hens and humans have

been shown to inhibit *in vitro* cell growth on chicken and human fibroblasts, respectively (Carrel and Ebeling, 1921; Baker and Carrel, 1925; Parker, 1931). Other reports showed no inhibitory effects on the cell growth by sera from old rabbit, rat, and human subjects (Kondo and Nomaguchi, 1985; Kondo *et al.*, 1988a, 1988b). Thus far, results have not been conclusive on age-related cellular factors in sera.

Schneider and Mitsui (1976) showed that the onset of cell senescence in human skin fibroblast cells derived from older (63 to 92 years) donors occurred more quickly than those from younger donors (21 to 36 years). Smith and Whitney (1980) reported that two human fibroblast cells derived from a single mitosis showed different population doubling capacity. Clark *et al.* (2003) reported that doubling capacity can vary between cell lines, and that genetics has been linked to this capacity. These studies illustrate the importance of the correct choice of cell line for NT.

The effects of animal sex on embryonic cell growth have been studied. Male human embryos consistently have more cells at the time of transplantation when compared with female embryos (Pergament *et al.*, 1994). These male conceptuses appeared to be healthier at the time of placement in the uterus (Pergament *et al.*, 1994). Fiddler *et al.* (1995) showed that the human Y chromosome associated SRY gene that encodes testis formation is expressed throughout the blastocyst stages and is active in stimulating cell division. The effect of activin-A on cell proliferation of differentiating rat gonad on days 14 and 15 postcoitum suggested that cell proliferation is differentially impacted depending on embryo sex (Kaipia *et al.*, 1994). Investigations of sex-related factors in mammalian cell lines have also been investigated using steroids, including estradiol and testosterone (Haug *et al.*, 1976; Kaipia *et al.*, 1994; Kwan *et al.*, 1996; Moraghan *et al.*, 1996; Mossuz *et al.*, 1998; Medina *et al.*, 2000; Griffin *et al.*, 2000; Ormerod *et al.*, 2003). In some reports sex effects were investigated in conjunction with parameters including cell byproducts or enzyme pathway alteration (Kwan *et al.*, 1996; Mossuz *et al.*, 1998).

According to these investigations on the impact of age and sex on mammalian cell growth, it is hard to conclude that there are no effects. If sex or age influences *in vitro* bovine cell growth (doubling capacity and/or mean cell generation time) or DNA synthesis, these parameters should be considered in screening donor cells for bovine NT. Thus, the main objective of this research is to investigate the effect of age and sex on the growth pattern of Angus (*Bos taurus*) cell lines as it relates to selecting a somatic cell line for NT.

## CHAPTER II

### LITERATURE REVIEW

The effect of age and sex on the growth pattern of the bovine cell lines was the focus of this study. To provide background about this thesis, research related to cell cycle and cell growth will be described. Factors relating to studies of cell cycle and cell growth include cell doubling capacity, cell cycle synchronization, flow cytometry analysis, and cryopreservation of cells. Next, growth factors in serum are discussed to introduce the effects of donor animal's age and sex as they relate to mammalian cell growth. Finally, significant developments in somatic cell nuclear transfer (NT) are illustrated.

#### **Cell Cycle and Cell Growth**

##### Cell Doubling Capacity

Eukaryotic cells grow in three phases in culture (Harrison and Rae, 1997). The lag phase follows reseeding and is prior to growth. Cell surface attachment starts and the logarithmic phase occurs with a population doubling period. The final, stationary phase, is characterized by slowing growth and maintenance of cells in a compact, confluent monolayer. Most cell types do not result in immortal cell lines, and after 50 doublings, die (Harrison and Rae, 1997). Subculturing can lead to chromosomal abnormalities such as dominant aneuploidies that negatively impact cell manipulations. Such considerations are important to nuclear transfer (NT) (Gómez *et al.*, 2003).

Primary cultures, derived from live animal tissues, are heterogeneous at the initial point of culture. With continuous *in vitro* culture, these cells become dominated by fibroblasts (Zimmermann *et al.*, 2001). Bovine fetal fibroblasts, often used in NT, have 30 to 50 population doublings prior to the onset of senescence (Polejaeva and Campbell, 2000). Schneider and Mitsui (1976) proved that the onset of senescence in human skin fibroblasts derived from older (63 to 92 years) people was quicker than that of younger donor's (21 to 36 years). Smith and Whitney (1980) reported that even two human fibroblast cell lines derived from a single mitosis showed different population doubling capacities. Nichols *et al.* (1977) found that the human diploid fibroblast-like cell strain IMR-90 derived from one female embryo showed three different doubling capacities depending on conditions including seeding density, growth medium, medium volume, and subcultivation reagents. However, Cristofalo *et al.* (1998) did not observe any relationship between donor age and replicative lifespan of human fibroblast cells. Clark *et al.* (2003) reported that the cell doubling capacity could vary widely between different

cell lines and thus genetics may have an important role in determining this capacity. This is important when selecting a cell line for NT.

#### Cell Cycle Synchronization

Cell proliferation is regulated by a number of factors. Extracellular conditions influence whether cells proceed with DNA synthesis or arrest in the G<sub>1</sub> phase (Mather, 1984). With appropriate DNA synthesis, each cell can progress through 4 cell cycle stages. A new eukaryotic cell first enters from G<sub>0</sub> phase (quiescent) to the G<sub>1</sub> phase (absence of DNA synthesis), then the S phase (DNA synthesis), then on to the G<sub>2</sub> phase (absence of DNA synthesis), with cell division occurring at the M phase (mitosis) (Hartwell *et al.*, 1974). If cells follow this pattern as a group *in vitro*, cultures are considered as synchronized (Cooper, 2003).

The cell cycle phase of donor nuclei is a very important factor to the success rate of the NT process (Campbell *et al.*, 1996). In the first production of a mammalian offspring from adult somatic cells, most of the donor cells are arrested in the quiescent stage (G<sub>0</sub>) by serum starvation (Wilmut *et al.*, 1997). In order to produce viable embryos (Campbell *et al.*, 1996; Collas *et al.*, 1992; Prather, 1996), most proposed that the donor nuclei should be in the G<sub>1</sub> or G<sub>0</sub> stage of the cell cycle when transferred to an oocyte cytoplasm with high levels of maturation promoting factor (MPF). Some studies indicated that donor somatic cells should be arrested in G<sub>0</sub> stage for successful cellular reprogramming, however, there were also reports of success in NT using cycling donor cells at the G<sub>1</sub> stage (Wells *et al.*, 1999; Cibelli *et al.*, 1998). Though some somatic cell cloning has been achieved with donor cells in G<sub>2</sub>/M phase (Cibelli *et al.*, 1998; Ono *et al.*, 2001), usually better efficiencies have been obtained with the use of G<sub>0</sub>/G<sub>1</sub> phase (Campbell *et al.*, 1996; Collas *et al.*, 1992; Prather, 1996).

To obtain cells synchronized at G<sub>0</sub>/G<sub>1</sub>, cultured cells are placed under certain conditions prior to NT. A primary way to synchronize the cells at the G<sub>0</sub>/G<sub>1</sub> phase is by serum starvation. Mammalian fibroblasts need mitogens to get through the G<sub>1</sub> phase. When cells pass the G<sub>1</sub> phase, they can enter the S phase and go through the cycle without further need of mitogens (Connell-Crowley *et al.*, 1998). The absence of mitogenic signals maintains the cell cycle at G<sub>0</sub>, characterized by low metabolic activity (Holley and Kiernan, 1968; Iyer *et al.*, 1999). A defect of serum starvation on cells in culture is that DNA fragmentation increases (Kues *et al.*, 2000; Peura, 2001; Gómez *et al.*, 2003). Alternative methods of synchronization include contact inhibition or reversible cycle inhibitors such as roscovitine, aphidicolin, and butyrolactone 1 (Wang, 1991;

Levenson and Hamlin, 1993; Kitagawa *et al.*, 1994; Boquest *et al.*, 1999; Kues *et al.*, 2000; Gibbons *et al.*, 2001). Aphidicolin is a reversible inhibitor of mammalian DNA polymerases and blocks the cell cycle at the transition from G<sub>1</sub> to S phase (Wang, 1991; Kues *et al.*, 2000). Butyrolactone 1 is an inhibitor of the cyclin-dependent kinase (Kitagawa *et al.*, 1994; Kues *et al.*, 2000). It arrests cell cycle at the transition from G<sub>1</sub> to S phase and at the transition from the G<sub>2</sub> to M phase (Kitagawa *et al.*, 1994). Butyrolactone 1 maintains the cell cycle in G<sub>1</sub> prior to phosphorylation of retinoblastoma protein (pRB), whereas, aphidicolin holds the cell cycle after pRB phosphorylation (Levenson and Hamlin, 1993; Kitagawa *et al.*, 1994; Kues *et al.*, 2000). When the plant amino acid mimosine was administered to Chinese hamster cell cultures for 14 hours after reversal of a G<sub>0</sub> block, it appeared to arrest the population at the G<sub>1</sub>/S boundary, and upon its removal cells entered the S phase in a synchronous wave (Mosca *et al.*, 1992).

Eighty percentage or higher synchronization into G<sub>0</sub>/G<sub>1</sub> phase synchronization were obtained in domestic cat fibroblasts at the arrested point by serum starvation, contact inhibition, and roscovitine treatment (Gómez *et al.*, 2003). In the case of porcine fetal fibroblasts, short times in (24 to 72 hours) serum deprivation significantly increased the proportion of cells at G<sub>0</sub>/G<sub>1</sub> phase to 77.9-80.2%, and mitotic activity had already been terminated after 48 hours and aphidicolin treatment led to an accumulation of  $81.9 \pm 4.9\%$  of cells at the G<sub>1</sub>/S transition (Kues *et al.*, 2000). Butyrolactone 1 arrested  $81.0 \pm 5.8\%$  of the cells at the end of G<sub>1</sub> stage (Kues *et al.*, 2000).

At a different point of view, Cooper (2003) reported that true cell cycle synchronization is impossible. Release of arrested cells from inhibition can not produce cells reflective of a normal division cycle. Cells arrested with a “G<sub>1</sub> phase amount of DNA” did not exhibit the expected DNA patterns after release from the cycle arrest (Di Matteo *et al.*, 1995; Cooper, 1998). However, even though perfect synchronization could not be obtained after the release from the cell cycle arrest, the importance of synchronization at the point of arrest have been validated with reports that cells arrested at G<sub>0</sub>/G<sub>1</sub> phases have been used for NT, whereby viable mammalian offspring have been produced (Collas *et al.*, 1992; Campbell *et al.*, 1996; Prather, 1996; Wilmut *et al.*, 1997).

### Flow Cytometry

The fluorescence activated cell sorter (FACS) was invented in the late 1960s by Bonner, Sweet, Hulett, Herzenberg and others to study viable cells (Hezenberg *et al.*,

2002). The ability to analyze and sort somatic cells is useful by enabling studies of cells that coexist in blood and various organs (Hezenberg *et al.*, 2002). The production of hybridomas by Kohler and Milstein in mid 1970s made the FACS and flow cytometry essential laboratory equipment (Kohler and Milstein, 1975).

Hybridomas produce specific monoclonal antibodies those are highly specific for their own antigens and can be conjugated to fluorescein, phycobiliproteins, and other fluorochromes (Hezenberg *et al.*, 2002). Analysis of the expression of genes is one of those methods (Aggarwal and Gupta, 1998). The expression of genes promoting apoptosis (*fas/fasL* and *bax*) and those inhibiting apoptosis (*bcl-2* and *bcl-x<sub>L</sub>*) in lymphocytes from aging and young subjects at the protein level were determined by flow cytometry (Aggarwal and Gupta, 1998).

Flow cytometric DNA analysis is a rapid and reliable method. It can be used with suspensions of fresh cells, fixed cells, or cells extracted from archival paraffin-embedded tissues from which histologic slides were made. The DNA content, or ploidy, of a cell population and the percentage of cells in S-phase can be determined. Flow cytometric cell cycle analysis of somatic cells established for bovine cloning was carried out (Kańska *et al.*, 2002). Cell cycle synchronization efficiency on porcine fetal fibroblasts (Kue *et al.*, 2000), African wild cat and domestic cat have also been investigated (Gómez *et al.*, 2003). Flow cytometry analysis of G<sub>0</sub> + G<sub>1</sub> phase has been successfully conducted with bovine cumulus and fibroblasts DNA cycle analysis and thus has been used as an effective tool for verifying the responsiveness of somatic cells used for donor nuclei in culture treatments (Kańska *et al.*, 2002).

### Cryopreservation

Freezing is a method to preserve cells long-term without genetic changes. Cryopreserved embryos can be transferred later to a foster mother and finally lead to term development of the embryo. There have been two primary cryopreservation advances for improved cell/embryo recovery including conventional slow cooling and vitrification.

A slow rate of cooling can be defined as slow and stepwise freezing method (Rall and Meyer, 1989). It attempts to maintain a delicate balance between the various factors, which may cause damage, such as ice crystal formation, osmotic injury, toxic effect of cryoprotectant, zona fracture of oocytes/embryos (Rall and Fahy, 1985; Rall and Meyer, 1989; Massip, 2001). To overcome cell damage during the freezing and thawing process, dimethyl sulfoxide (DMSO) and glycerol have been used as

cryoprotectants (Mazur, 1970; Lovelock and Bishop, 1959; Kelbe and Mancuso, 1983). Mammalian cell lines can be stored in 10% DMSO with the desired growth medium either with or without fetal bovine serum (FBS) (Corsini *et al.*, 2004).

Vitrification was first introduced with mouse embryos as a new method of cryopreservation of mammalian embryos in the absence of ice. It is a physical process by which a solution is transformed into a stable glass by rapid cooling, bypassing ice crystal formation while maintaining the properties of a liquid in a solid form (Rall and Fahy, 1985). When the environment changes into a vitreous (glasslike) state, cells can be cryopreserved. Briefly, vitrification is a thermodynamic state of metastable equilibrium. This methodology, which is based on straws with a narrow diameter that increases surface area to volume ratio, was developed to increase cooling rates of media during vitrification (Vajta *et al.*, 1998). Vitrification by the open pulled straw method was reliable and effective for the cryopreservation of human pluripotent embryonic stem cells (Reubinoff *et al.*, 2001).

Over the past 15 years, advances both in cryopreservation of embryos and cells, as well as their analysis by flow cytometry, have allowed NT to progress. In depth studies employing these biotechnologies has supported such studies as cell line maintenance (Nichols *et al.*, 1977; Smith and Whitney, 1980; Cristofalo *et al.*, 1998; Clark *et al.*, 2003), synchronization (Gómez *et al.*, 2003; Kues *et al.*, 2000; Cooper, 2003; Di Matteo *et al.*, 1995; Cooper, 1998), increasing NT efficiency by studies of donor animal nuclei (Collas *et al.*, 1992; Campbell *et al.*, 1996; Prather, 1996) and recovery of genetic cell lines (Rall and Fahy, 1985; Reubinoff *et al.*, 2001; Corsini *et al.*, 2004).

## **Growth Factors in Serum**

### Polypeptide Growth Factors

Complex signaling pathways involved with cell growth, differentiation, migration, and apoptosis are regulated partly by polypeptide growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and epidermal cell derived factor (EDF). These growth factors in serum can act as positive or negative modulators (Favoni and de Cupis, 2000). Epidermal growth factor (EGF), in particular, has been found to play an important role in stimulating cell proliferation, and differentiation (Adamson, 1990).

Recently epidermal cell derived factor (EDF), present in the supernatant of cultured epidermal cells, was found to stimulate the growth of keratinocytes and inhibit fibroblast proliferation (Eisinger *et al.*, 1988). In fibroblast cultures, EDF inhibited the



ability of fibroblasts to cause contraction of collagen sponges by 90% (Eisinger *et al.*, 1988). Application of EDF to surgical wounds stimulated extensive migration and proliferation of keratinocytes which can be differentiated to epidermis (Eisinger *et al.*, 1988).

Though polypeptide growth factors have been suggested as primary effectors on cell growth, there have been other factors that should be considered including age-related factors and sex-related factors.

#### Age-related Factors and Their Influence on Cell Growth

Investigations on the effects of sera derived from animals of different ages on cell growth in culture have been carried out, however, results are not conclusive (Carrel and Ebeling, 1921; Baker and Carrel, 1925; Parker, 1931; Kondo *et al.*, 1988). Several studies have been done on inhibitory factors in sera derived from old hens and humans. Cell growth of chicken fibroblasts and human fibroblasts were inhibited (Carrel and Ebeling, 1921; Baker and Carrel, 1925; Parker, 1931). However, sera from old rabbits (5 to 7.8 years) increased rabbit fetal skin fibroblast proliferation more than sera from 8 month old rabbits (Kondo and Nomaguchi, 1985). Most samples of sera from older rats (even those 29 months old) stimulated cell growth (rat fetal skin fibroblast) as well as those from younger (3 to 12 months) ones (Kondo *et al.*, 1988a). Sera from old male or female humans (up to 80 years old) did not inhibit the proliferation of human fetal lung fibroblast (TIG-1) cells (Kondo *et al.*, 1988b).

Human sera of both sexes from subjects in their 60s (60 to 64 years) were more inhibitory (8 to 14%) to the migration of human fetal lung fibroblasts than serum from subjects in their 20s (20 to 29 years), suggesting that human sera from older subjects contained substances inhibitory to cell migration (Kondo *et al.*, 1989). Thus far, the results on these age-related cellular inhibitory factors in sera have been inconclusive.

In addition to age-related effects on cell growth induced by serum factors, cells derived from animals of various ages proliferate differently. Proliferation of rat aortic smooth muscle cells was investigated as a function of age (Nakao *et al.*, 1984). Lower migratory patterns of older human fibroblast lines were seen compared with that from younger donors (Kondo and Yonezawa, 1992). In rat foot epidermis, the duration of G<sub>1</sub> stage duration was shorter in 7-week-old rats ( $15.0 \pm 0.8$  hours) than in 52-week-old rats ( $31.2 \pm 3.5$  hours) (Morris *et al.*, 1990). Age-related changes in the mitotic and metabolic characteristics of rat muscle-derived cells (mdc) also have been reported (Barani *et al.*, 2003). Muscle-derived cells were isolated from gastrocnemius and quadriceps muscles

of young (3 weeks), adult (9 months), and old (24 months) male Sprague-Dawley rats (n=10/group) (Barani *et al.*, 2003). The mdc from young rats proliferated earlier compared with those from older animals (Barani *et al.*, 2003). Plasminogen activators and matrix metalloproteinase activities were significantly decreased in cells from older rats (Barani *et al.*, 2003). These results showed that cellular and biochemical events related to the control of mdc activation and proliferation change with aging and these changes cause incomplete repair and contribute to the loss of skeletal muscle mass and function with aging (Barani *et al.*, 2003).

There have been numerous reports on wound repair related to cell age (Goodson and Hunt, 1979; Muggleton-Harris *et al.*, 1982; Cohen *et al.*, 1987; Holt *et al.*, 1992). Response to cell sheet wounding in confluent cultured normal human skin fibroblasts from neonatal, adult and aged donors has been studied (Muggleton-Harris *et al.*, 1982). The latent period (time between the start of wounding and initiation of migration from the edge of the monolayer) was positively correlated with donor's age (Muggleton-Harris *et al.*, 1982). The healing responses in human subjects *in vivo* has also been investigated (Grove, 1982). The reestablishment of skin surface markings in unroofed blisters made by exposure to a 1:1 aqueous solution of ammonium hydroxide was monitored. At all stages of repair, older individuals (65 to 75 years) lagged behind young adults (18 to 25 years) (Grove, 1982). The closure of bilateral, full-thickness cutaneous wounds made over the back with a sharp paper punch was measured with calipers and assessed histologically in C57BL/6J male mice for 10 days after wounding (Cohen *et al.*, 1987). Mice at 6 months of age exhibited more rapid wound closure and repair than did mature (15 months) or aged (26 or 27 months) mice (Cohen *et al.*, 1987). In humans, aging leads to delayed epithelialization on wound healing. The elderly subjects (more than 65 years old) showed a delay of 1.9 days in epithelialization compared to others (18 to 55 year old healthy subjects) (Holt *et al.*, 1992).

#### Sex-related Factors and Their Influence on the Cell Growth

Serum and the characteristics of cells related to the sex from which they were derived should be considered simultaneously when examining their influence on cell growth. Investigations on sex-related factors have focused primarily on steroids or on specific glycoproteins (Haug *et al.*, 1976; Faulkner *et al.*, 1989; Kaipia *et al.*, 1994; Kwan *et al.*, 1996; Moraghan *et al.*, 1996; Mossuz *et al.*, 1998; Medina *et al.*, 2000; Griffin *et al.*, 2000; Ormerod *et al.*, 2003). The influence of activin-A on cell proliferation of differentiating rat gonad showed sex can influence proliferation in certain cell types

(Kaipia *et al.*, 1994). Activin stimulated thymidine incorporation in ovaries and female mesonephroi on days 14 and 15 postcoitum in a dose-dependent manner. However, activin inhibited thymidine incorporation in testes and male mesonephroi on day 14 postcoitum in a dose-dependent way (Kaipia *et al.*, 1994).

The sex hormone estradiol controls collagen synthesis in the mesangial cell (derived from the kidney of male Sprague-Dawley rats) while testosterone does not affect the collagen synthesis of mesangial cells (Kwan *et al.*, 1996). Mossuz *et al.* (1998) reported that the growth pattern of some human leukemic cells may be inhibited by sex steroids (estradiol and testosterone), independently of nuclear steroid receptor expression.

Gender-related differences on the effects of estrogen under hypoxic conditions have yielded important results (Griffin *et al.*, 2000). In female human cardiac fibroblasts, DNA synthesis remained unchanged under hypoxic conditions (Griffin *et al.*, 2000). Male cells, on the other hand, were susceptible to hypoxia and their DNA synthesis was significantly decreased (70%,  $P < 0.0001$ ) (Griffin *et al.*, 2000). The presence of estrogen altered these responses to hypoxia in both male and female cells (Griffin *et al.*, 2000). In female cells, combined effects of hypoxia and estrogen led to the inhibition of DNA synthesis, whereas in male cells estrogen partially reversed the hypoxia-induced inhibition of DNA synthesis (37% with estrogen versus 70% without estrogen). Thus, estrogen was proven to differentially alter the responses of male and female cells to hypoxia via an estrogen-receptor-dependent mechanism. (Griffin *et al.*, 2000).

As the technique of *in vitro* fertilization (IVF) became more wide spread in the early 1990s, women undergoing IVF were reported to be giving birth to more male babies than female (Pergament *et al.*, 1994). Male embryos progressed through the second cell division faster than female embryos (Ursula Mittwoch, 1988; Pergament *et al.*, 1994) and continue to develop more rapidly than female embryos throughout early development (Pergament *et al.*, 1994). Because male embryos consist of more cells per embryo at the time of surgical implantation, these male embryos might appear to be healthier and therefore selected for transplantation into the uterus (Pergament *et al.*, 1994). Fiddler *et al.* (1995) have shown that the human SRY gene, which is on the Y chromosome and encodes the major determinant of testis formation later in development, was expressed during the zygote stage and throughout the blastocyst stages and active in stimulating cell division.

In several reports on wound repair related to sex (Ashby *et al.*, 1969; Liu *et al.*, 2001; Hawkey *et al.*, 2002), no differences in healing rates were noted for peritoneal tissue in male and female rats (Ashby *et al.*, 1969). However, female rats with gastritis had a faster ulcer-healing rate than males (Liu *et al.*, 2001). Finally, healing of gastric erosions derived from *Helicobacter pylori* infections were faster in males after non-steroidal anti-inflammatory drug treatment in human subjects (Hawkey *et al.*, 2002).

### **Somatic Cell Cloning**

In 1997, “Dolly” was created from the transfer of a nucleus of a differentiated somatic cell into an enucleated oocyte by the process of NT (Wilmut *et al.*, 1997). Since the birth of a cloned sheep numerous other mammalian species have been cloned including the cow (Cibelli *et al.*, 1998), mouse (Wakayama *et al.*, 1998), goat (Baguisi *et al.*, 1999), pig (Polejaeva *et al.*, 2000; Betthausen *et al.*, 2000), domestic cat (Shin *et al.*, 2002), mule (Woods *et al.*, 2003), horse (Galli *et al.*, 2003), and rat (Zhou *et al.*, 2003). Somatic cell NT is a method to develop specific animal genotypes, and its usage has been expanded to transgenic (Baguisi *et al.*, 1999) livestock, which can be used as tools of the pharmaceutical industry (Ziomek, 1998).

Though animal clones from somatic cells have been developed in many species, overall efficiency was less than 1% of the reconstructed embryos (Han *et al.*, 2003). This low efficiency as well as high cost are disadvantages to this research area. Low efficiencies have been attributed to early stage embryo death categorized as circulatory distress, placenta edema, hydrallantois, and chronic pulmonary hypertension, large placenta, large offspring syndrome, immune dysfunction of kidney, and brain malformation (Wakayama *et al.*, 1998; McCreath *et al.*, 2000; Eggan *et al.*, 2001; Ono *et al.*, 2001; Han *et al.*, 2003). These abnormalities have been explained in three ways; 1) aberrant epigenetic reprogramming in nuclear transfer (NT) embryos (Kang *et al.*, 2001), 2) aberrant epigenetic reprogramming between inner cell mass (ICM) and trophectoderm (TE) cells (Wakayama *et al.*, 1998), and 3) structural abnormalities (the ratio of cell numbers between ICM and TE) (Koo *et al.*, 2002).

The aberrant epigenetic reprogramming in whole embryos can be defined as differential demethylation, which appears among different genomic sequences in NT embryos. According to Kang *et al.* (2001), the degree of methylation of bovine satellite sequences was varied in each NT embryo and only 26% (7/27) were undermethylated, though their methylation levels were higher than the normal control embryos.

In contrast, aberrant epigenetic reprogramming between ICM and TE cells in NT embryo has been demonstrated. Though the mechanism related to a difference in methylation status of the ICM and TE cells of the normal blastocyst were not clearly revealed, methylation level increases in fetus proper (primitive ectoderm lineage), whereas, methylation levels remained low throughout gastrulation as if *de novo* methylation has not been accomplished in mice in extraembryonic tissues come from TE or primitive endoderm lineages (Chapman *et al.*, 1984; Rossant *et al.*, 1986). Therefore, the placental dysfunction was the likely phenotype that could be explained by the epigenetic anomaly of TE cells. As a deficient placentation is frequently observed in dead fetal clones of various mammalian species and neonatal mortality in cloned animals (Wakayama *et al.*, 1998), the correlation of abnormal methylation in TE cells of NT blastocysts with the placental defects investigated in NT fetuses should not be ignored.

A third explanation of early fetal loss is structural anomalies of early NT embryos. The NT blastocysts have shown a significantly higher proportion of ICM cells than IVF- and *in vivo*-derived embryos (Koo *et al.*, 2002). These results indicate that structural anomalies, which can affect the survival of NT embryos after transfer, may arise during preimplantation development.

To lessen the occurrence of such process that result in abnormalities due to the NT process and to achieve more efficient NT, systematic strategies have been suggested. Nagy *et al.* (1990) first developed the tetraploid complementation concept. Several newly generated mice embryonic stem (ES) cell lines were tested for their ability to produce completely ES cell-derived mice at early passage by ES cell ↔ tetraploid embryo aggregation. One cell line produced live offspring, which were completely ES-cell derived as judged by isoenzyme analysis and coat color. These animals were normal, viable and fertile (Nagy *et al.*, 1990).

A few years later, some specific genes important to the NT process have been revealed (Nagy *et al.*, 1993; Guillemot *et al.*, 1994). In *Mash-2*<sup>-</sup> mutants, only trophoblast cells in the embryos displayed obvious developmental defects (Guillemot *et al.*, 1994). The placental phenotype of the *Mash-2*<sup>-</sup> mutation was rescued by aggregating *Mash-2*<sup>-/-</sup> diploid and wild-type tetraploid murine embryos (Nagy *et al.*, 1993). The essential role of the gene *Mash-2* was reported to support tetraploid complementation (Guillemot *et al.*, 1994). Embryo death at about 10 days post-coitum is also consistent with a primary placental failure (Guillemot *et al.*, 1994). Results showed that *Mash-2*<sup>-/-</sup> cells can support

embryonic development, yet *Mash-2* has no obvious embryonic function (Guillemot *et al.*, 1994). Using *Hnf-4*<sup>-/-</sup> ES cells, Duncan *et al.* (1997) demonstrated that *Hnf-4* is a key regulator of tissue-specific gene expression in murine visceral endoderm (VE), which is essential for the normal expression of factors including alphafetoprotein, apolipoproteins, transthyretin, retinol binding protein, and transferrin. This report suggested that the specific complementation of *Hnf-4*<sup>-/-</sup> embryos with tetraploid-derived *Hnf-4*<sup>+/+</sup> VE could rescue early developmental arrest (Duncan *et al.*, 1997).

Live calves have been produced from tetraploid embryo complementation to the embryonic stem-like cells (Iwasaki *et al.*, 2000). Transgenic mouse embryos were generated directly from embryonic stem cells by tetraploid embryo complementation (Misra *et al.*, 2001). Eggan *et al.* (2001) reported that tetraploid embryo complementation in mice using F<sub>1</sub> embryonic stem cells represent a simple and efficient procedure for deriving animals with complex genetic alterations without the need for chimeric intermediates. Male and female mice derived from the same embryonic stem cell clone by tetraploid complementation have also been produced (Eggan *et al.*, 2002).

Further advances have been made using ICM transplantation methodologies (Polzin *et al.*, 1987; Rorie *et al.*, 1994). Direct interspecific and intergeneric embryo transfers have been attempted between domestic animals and other related wild species, yet viable offsprings, embryo survival rate to term have often been low (Lasley *et al.*, 1994). Intergeneric embryo transfer results were even less successful and rarely results in live young (Anderson, 1988; Lasley *et al.*, 1994). These failures can be explained as physiological differences in fetal-maternal interaction (MacLaren *et al.*, 1992) and immunological rejection of the conceptus (Anderson, 1988). Polzin *et al.* (1987) tried to improve on this concept and reported sheep-goat chimeras by ICM transplantation. In the experiments, goats ICM was injected into ovine blastocysts using micromanipulators. Twenty-two manipulated blastocysts were surgically transferred into 12 ovine recipients. Nine ewes gave birth to a total of 13 young. Ten were classified by serum electrophoretic assays or karyotypes as lambs, one as a kid, and two as interspecific chimeras (Polzin *et al.*, 1987). Rorie *et al.* (1994) refined the methodology and demonstrated simultaneous substitution of extrinsic ICM with the intrinsic ICM. In the case of ICM transplantation by Polzin group, the ICM from the original blastocyst was allowed to aggregate with injected ICM. The sheep ICM was transplanted into the goat blastocyst and simultaneously the original goat ICM was removed (Rorie *et al.*, 1994). Nine such reconstructed blastocysts were transferred into synchronous recipient does,

and twin ewe lambs were born after 148 days of gestation (Rorie *et al.*, 1994). This ICM transplantation method coupled with compensatory techniques to decrease trophoctoderm abnormalities, further advanced the success of NT.

Up to now, from studies on the cell cycle to the development of somatic cell NT technologies, much research has been done. Through literature reviews, the importance of choosing donor cell line for NT has been suggested to be as important as the development of somatic cell NT methodologies themselves. Even though good methodologies have been developed, if the criteria in choosing donor cell lines are not well established, all NT processes thereafter would be affected. From the desirable choice of cell lines to the sophisticated NT techniques, further progress in somatic cell NT can be anticipated with future research.

### CHAPTER III

## THE EFFECT OF AGE AND SEX ON THE GROWTH PATTERN OF FRESH BOVINE CELL LINES

### Introduction

Following the 1997 birth of “Dolly” the sheep that was created from the transfer of a nucleus from a differentiated somatic cell into an enucleated oocyte (Wilmut *et al.*, 1997), individuals from numerous mammalian species have been cloned (Cibelli *et al.*, 1998; Wakayama *et al.*, 1998; Baguisi *et al.*, 1999; Betthausen *et al.*, 2000; Shin *et al.*, 2002). The overall efficiency of nuclear transfer (NT) has been less than 1% of reconstructed embryos (Han *et al.*, 2003), and is coupled with high costs and tedious methods. Cloned animal deaths are sometimes due to circulatory distress, placental edema, hydrallantois, chronic pulmonary hypertension, large placentae, large offspring syndrome, immune dysfunction of kidney, and brain malformation (Wakayama *et al.*, 1998; McCreath *et al.*, 2000; Eggan *et al.*, 2001; Hill *et al.*, 2000; Ono *et al.*, 2001; Han *et al.*, 2003).

Abnormalities in NT embryos may be caused by aberrant epigenetic reprogramming (Kang *et al.*, 2001), aberrant epigenetic reprogramming between inner cell mass (ICM) and trophectoderm (TE) (Wakayama *et al.*, 1998), or structural abnormalities as shown by cell ratios of ICM and TE cell numbers (Koo *et al.*, 2002). To overcome such developmental problems, tetraploid complementation (Nagy *et al.*, 1990; Duncan *et al.*, 1997; Eggan *et al.*, 2002) or ICM transplantation have been adopted to supplement trophectoderm growth (Polzin *et al.*, 1987; MacLaren *et al.*, 1992; Rorie *et al.*, 1994).

Additionally donor cell culture systems have been enhanced. Specifically, the DNA cell cycle phase of donor nuclei has been found to be of primary importance to the success of NT and embryo reconstruction (Wang, 1991; Mosca *et al.*, 1992; Kitagawa *et al.*, 1994; Wilmut *et al.*, 1997; Cibelli *et al.*, 1998; Cooper, 2003).

A limited number of studies have been conducted on the impact of animal age on the growth of cells during *in vitro* culture, such as the influence of sera derived from animals of different ages. Sera from old hens (3 to 9 years) inhibited the growth of chicken fibroblasts compared with those of young chickens (3 weeks to 6 months) (Carrel and Ebeling, 1921; Baker and Carrel, 1925). Sera from adult humans (27 years) inhibited the growth of human fibroblasts in culture compared with sera from infants (14 months) (Parker, 1931).

In another report on humans, sera from each sex of subjects in their 60s (60 to 64 years) were more inhibitory (8 to 14%) to the migration of human fetal lung fibroblasts than



sera derived from subjects between 20 and 29 years, implying serum inhibitory factors to cell migration were present in aged humans (Kondo *et al.*, 1989). However, there have been contradictory reports. Sera from old rabbits (5 to 7.8 years) significantly increased the proliferation of cells as compared with serum from 8 month old rabbits (Kondo and Nomaguchi, 1985). Sera from old rats (25 to 29 months) stimulated cell growth (rat fetal skin fibroblast) as well as those from younger rats (2 to 12 months) (Kondo *et al.*, 1988a). In contrast, sera from old men and women (up to 80 years old) did not inhibit the proliferation of human fetal lung fibroblasts (TIG-1) (Kondo *et al.*, 1988b). These examples and those of others indicate that the evidence is inconclusive regarding age-related extracellular serum factors and their impacts on growth of cells in culture.

Investigations have been conducted on the impact of cell characteristics on their growth in culture. The decreased proliferation of rat aortic smooth muscle cells was seen with increasing age of cells (Nakao *et al.*, 1984). Human skin fibroblasts from adult donor migrate slower than fetal skin fibroblasts (Kondo and Yonezawa, 1992). The time of G<sub>1</sub> duration of rat foot epidermis was shorter in 7-week-old rats than that of 52-week-old rat (Morris *et al.*, 1990).

Investigations have been conducted on wound repair as related with cell age (Goodson and Hunt, 1979; Grove, 1982; Muggleton-Harris *et al.*, 1982; Cohen *et al.*, 1987; Holt *et al.*, 1992). Confluent normal human skin fibroblasts from neonatal, adult and aged donors were stimulated to respond to wounding of the cell sheets *in vitro*. After wounding, the latent period (time prior to the initial migration of cells from the edge of the monolayer) was positively correlated to donor age (Muggleton-Harris *et al.*, 1982). It has been found that 6-month-old mice exhibited more rapid rate of wound closure and repair than did mature (15 months) or aged (26 or 27 months) mice (Cohen *et al.*, 1987). At each stage of wound repair, older humans (65 to 75 years) lagged behind young adults (18 to 25 years) (Grove, 1982). Elderly subjects (more than 65 years old) showed delays of 1.9 days longer in epithelialization than did other healthy subjects (18 to 55 years) (Holt *et al.*, 1992).

The impact of sex on wound repair has also been studied (Ashby *et al.*, 1969; Liu *et al.*, 2001; Hawkey *et al.*, 2002). When peritoneal healing rates in male and female rats were compared no difference was detected (Ashby *et al.*, 1969).

Female rats with gastritis were found to have faster ulcer-healing rates than males (Liu *et al.*, 2001). Healing rates of gastric erosion from *Helicobacter pylori* infections for

women were faster than those of males after non-steroidal anti-inflammatory drug treatments (Hawkey *et al.*, 2002).

Additionally, reports have been published on factors relating to impact of sex on cell proliferation (Faulkner *et al.*, 1989; Kaipia *et al.*, 1994; Kwan *et al.*, 1996; Griffin *et al.*, 2000). Crossbred yearling heifers implanted with testosterone propionate increased daily weight gain ( $P < 0.05$ ) and feed efficiency compared with heifers without implants (Faulkner *et al.*, 1989). Exposure of differentiating rat gonadal cells to activin-A showed sex can influence proliferation in certain cell types within the gonad (Kaipia *et al.*, 1994).

Estradiol was shown to influence collagen synthesis of mesangial cell (derived from the kidney of male Sprague-Dawley rats), while testosterone had no effect (Kwan *et al.*, 1996). Hence, studies were carried out on gender-related differences and the effect of estrogen under hypoxia. Estrogen was proven to differentially alter the responses of human male and female fibroblasts to hypoxia via estrogen-receptor-dependent mechanisms (Griffin *et al.*, 2000).

This study evaluated the choice of donor cells in an effort to increase subsequent NT efficiency. It is proposed that sex and/or age of the cell donor animal influences cell population development in culture. The hypothesis is that the sex and age of donor animals from which somatic cells were derived influences cell growth *in vitro*. The specific objective was to investigate growth patterns of bovine (*Bos taurus*) cells of various ages and sexes in early stages (passage 2) of *in vitro* growth. Such information could provide information to help select donor somatic cells for subsequent use in nuclear transfer.

## **Materials and Methods**

### **Experimental Design**

Four different animals were assigned to 4 treatment groups by sex and age in a 2 x 2 factorial arrangement (Table 1). Adult males ranged from 1.70 to 4.25 years of age (average =  $2.73 \pm 0.63$  years). Young males ranged from 0.20 to 0.30 years of age (average =  $0.25 \pm 0.02$  years). Adult females ranging from 3.30 to 8.50 years old (average =  $5.08 \pm 1.16$  years). Young females ranged from 0.20 to 0.30 years of age (average =  $0.23 \pm 0.01$  years).

The body condition scores (Short *et al.*, 1990; Erb *et al.*, 1990) ranged from 5 to 6. The Male Calves (MC) and Female Calves (FC) groups were composed of healthy calves with body weights ranging between 110 and 190 kg. There were no body condition scores for Male Calves (MC) and Female Calves (FC).

### Sampling Animals

Skin biopsies were obtained from four Angus (*Bos taurus*) animals per group with groups defined as Bulls (B), Cows (C), Male Calves (MC), and Female Calves (FC) maintained at the Purebred Beef Unit of Louisiana State University in June of 2004 (Table 1). Cell populations were identified by the ear tag identification number of each animal in this study. Skin tissues were taken from the neck region of each animal. Specifically, 5 cm above the jugular vein was the site for the skin biopsy. The area biopsied was shaved, cleaned with betadine scrub and then washed with 70% ethanol three times. A 2-mm biopsy punch (Miltex, Bethpage, NY) was used to obtain two samples of skin from each animal. The samples were immediately transferred to sterile 15-ml centrifuge tubes (Corning, New York, NY) prefilled with phosphate-buffered saline (PBS), 100 µg/ml kanamycin (Sigma, Grand Island, NY), and 100 µg/ml gentamycin (Gibco, St. Louis, MO).

### Processing Tissue to Obtain Cell Lines

The skin biopsies in 15-ml centrifuge tubes were transferred to 35 x 10 mm Falcon™ plastic Petri dishes (Becton and Dickinson, Lincoln Park, NJ), and sterile iris scissors and sterile forceps were used to separate the epidermal layer from connective tissue. The epidermal layer was then placed in another 35 x 10 mm Petri dish and minced into a paste with iris scissors with 100 µl of modification #2 of Dulbecco's modified eagle medium (M2DMEM). The M2DMEM was composed of DMEM (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 100 µg/ml kanamycin, and 100 µg/ml gentamycin. Then, 900 µl of M2DMEM was added and the suspension was transferred to a 15-ml tube and 3 ml of M2DMEM was added to wash the minced tissue. The mixture was gently swirled and held for 5 minutes until a pellet formed. The supernatant was removed and 3 ml of M2DMEM was added to wash the tissue. This washing process was repeated two more times. After washing, the pellet was transferred into 900 µl of M2DMEM in a 35 x 10 mm Petri dish, and incubated in 5% CO<sub>2</sub> in air at 38°C. Cell growth was examined using phase contrast microscopy on an inverted microscope (Diaphot, Nikon, Garden, NY).

The medium was replaced with 2 ml of modification #1 of DMEM (M1DMEM) every three days after the 5<sup>th</sup> day of *in vitro* culture. The M1DMEM was composed of DMEM (Gibco) with 10% FBS, 50 µg/ml of kanamycin and 50 µg/ml of gentamycin. When the attached cells reached more than 50% confluency, the original tissue mass was removed.

Table 1. The experimental design and characteristics of Angus bulls, cows, female calves, and male calves used in this study

Group	Sex	ID	Age (yr)	Birth	Weight (kg)	BCS*
<b>Bulls (B)</b>	M	110	4.25	Jan/28/00	841.2	5.0
		1014	3.25	Jan/22/01	845.8	6.0
		2507	1.70	Sept/22/02	508.4	5.5
		2505	1.70	Sept/17/02	479.3	5.0
		Mean±SE	2.73±0.63		668.7±101.1	5.4±0.2
<b>Male calves (MC)</b>		4020	0.30	Jan/26/04	179.6	
		4044	0.25	Feb/16/04	111.6	
		4033	0.25	Feb/05/04	190.5	
		4090	0.20	Mar/15/04	121.5	
		Mean±SE	0.25±0.02		150.8±20.0	
<b>Cows (C)</b>	F	6064	8.50	Nov/11/96	628.7	6.0
		580	4.25	Feb/22/00	553.3	5.0
		670	4.25	Feb/24/00	509.7	5.0
		1027	3.30	Jan/31/01	539.7	6.0
		Mean±SE	5.08±1.16		557.7±25.2	5.5±0.3
<b>Female calves (FC)</b>		4035	0.25	Feb/07/04	117.9	
		4076	0.20	Mar/05/04	110.7	
		4075	0.20	Mar/05/04	127.0	
		4060	0.25	Feb/27/04	129.2	
		Mean±SE	0.23±0.01		121.2±4.3	

\*BCS (body condition score) for bulls and cows were the courtesy of Ray Shields at LSU Purebred Beef Unit.

### Maintenance of Cell Lines

Each of the 16 cell lines was cultured in 5% CO<sub>2</sub> in air in a 38°C incubator (Series II water jacketed CO<sub>2</sub> incubator, Thermoforma, Marietta, OH). When the primary culture reached 100% confluency, cells were exposed to trypsin-EDTA (Sigma) and viable cell counts were made with a hemocytometer (Cambridge, Buffalo, NY) and 0.4% trypan blue (Sigma). For the first passage, 2 ml of 5 x 10<sup>4</sup> cells/ml was used as an initial seeding in M1DMEM in a 35 x 10 mm Petri dish. When the culture reached a confluent monolayer, 3 to 4 days of contact inhibition was used for inducing cell cycle synchronization.

### Growth Curve

For passage 2, 1 ml of cells (1 x 10<sup>4</sup>/ml) was used for the initial seeding in M1DMEM in 1 well of a 4-well dish (Nunc, Roskilde, Denmark). The surface area of 1 well of a 4-well plate was 1.13 cm<sup>2</sup>. On days 1, 2, 4, 6 and 8, cells were obtained for viable cell counting. Cells were washed with DMEM and trypsinized using 700 µl of trypsin-EDTA (Sigma) for 5 minutes in 5% CO<sub>2</sub> at 38°C. The trypsinized cells were transferred to a 1.5 ml microfuge tube and centrifuged at 400 x g for 4 minutes. Viable cell counts of each sample in triplicates were carried out.

### Mean Generation Time and Other Growth Parameters

The mean cell generation time (MGT) of each cell line was calculated using an equation in conjunction with plots depicting logarithmic phases of growth (Dawes, 1980) (Figure 1). The interval from the start of cell seeding into culture to the start of their logarithmic phase of growth or lag time was recorded. The viable cell counts obtained from the stationary phase of growth or stationary phase viable cell counts (SPCC) were recorded for each cell line in this study. These three parameters have been used in quantitative evaluation of cell growth patterns described previously (Mangels *et al.*, 1978; Hutson *et al.*, 1988).

### Familial Lineage and Growth Parameters

In this study, a familial lineage relationship figure was prepared for the donor animals (Figure 2). Mean cell generation times (MGT) were compared among individuals within a group, and between all individuals in all groups. A rank from 1<sup>st</sup> to 4<sup>th</sup> was assigned to individuals within each group, with shorter MGT receiving lower ranks. Ranked individuals were assigned to categories, with the first category I containing 1<sup>st</sup> to 4<sup>th</sup> ranks, category II consisting of 5<sup>th</sup> to 8<sup>th</sup> ranks, category III consisting of 9<sup>th</sup> to 12<sup>th</sup>, and category IV consisting of 13<sup>th</sup> to 16<sup>th</sup>. Again shorter MGT were in categories ranked lower (I < IV).

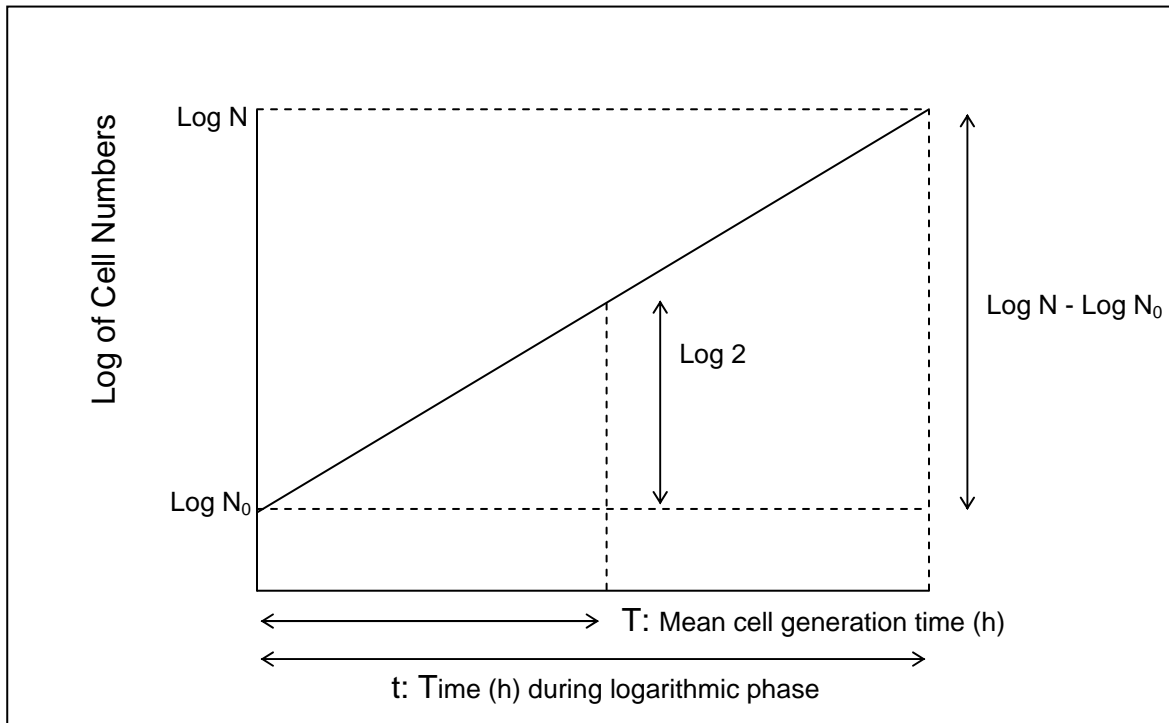


Figure 1. A logarithmic plot for calculating mean generation time of cells in culture (Dawes,1980).

$$(\text{Log } N - \text{Log } N_0)/t = (\text{Log } 2)/T.$$

N Number of cells in stationary phase.

N<sub>0</sub> Number of cells at the beginning of logarithmic phase.

t Time (h) during logarithmic phase.

T Mean cell generation time.

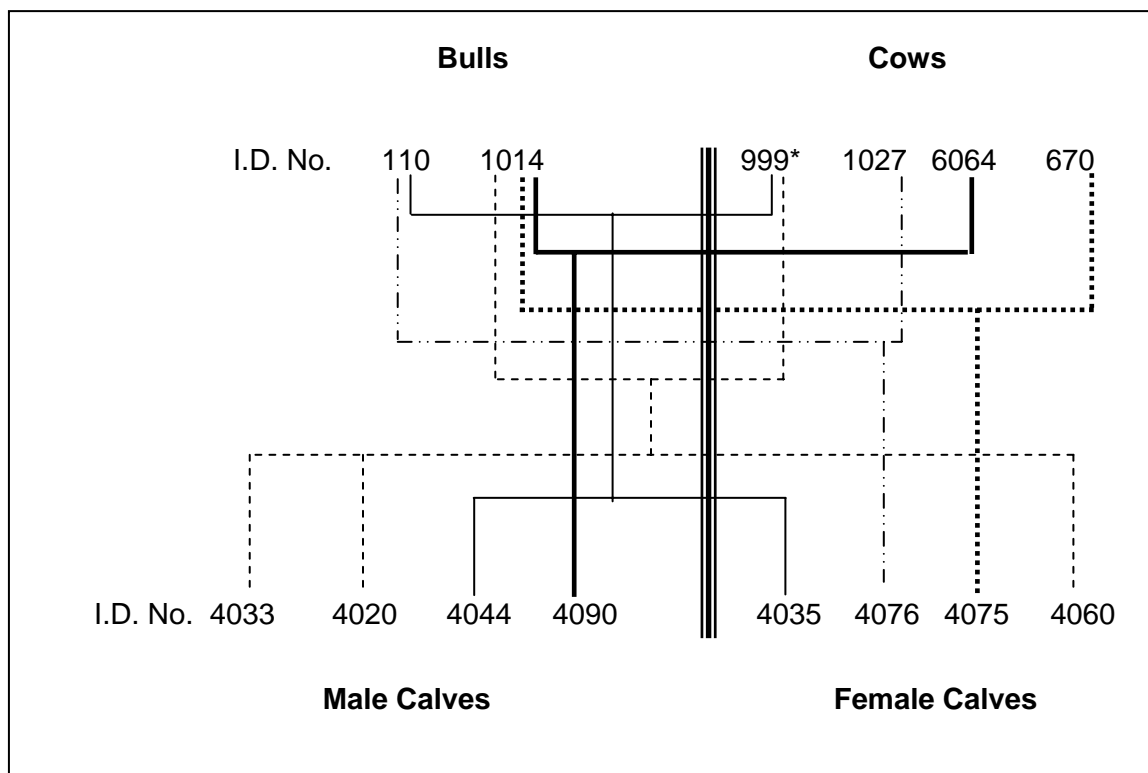


Figure 2. Familial lineage depicting breeding scheme and bovine cell line identification number. Bull #110 x cow #999 produced male calf #4044 and female calf #4035. Bull #110 x cow #1027 produced female calf #4076. Bull #1014 x cow #999 produced male calves #4033, #4020 and female calf #4060. Bull #1014 x cow #6064 produced male calf #4090. Bull #1014 x cow #670 produced female calf #4075. \*Cows #999: No mean cell generation time data on these cows were obtained in this study.

In time of analysis, the MGT with the 1<sup>st</sup> or 2<sup>nd</sup> rank was accepted as comparatively short MGT within group against the 3<sup>rd</sup> or 4<sup>th</sup> rank. The MGT in category I or II over all groups was regarded as comparatively short MGT over those of the other categories (III or IV). According to the familial lineage (Figure 2), each cell line bull #1014, bull #110, cow #6064, and cow #670 and their respective offspring were grouped as “related”, while other animals were grouped as “unrelated” (i.e., bull #1014 related versus bull #1014 unrelated). Lag time, SPCC, and MGT were compared between related and unrelated groups.

#### Statistical Analysis

Data on lag time, SPCC, and MGT were analyzed by ANOVA, with sex, age and their interactions in the model. Then, each group was compared with another group in the factorial arrangement. For the pairwise analysis, eight pairs of comparisons were evaluated. For the comparison of related animal effect, individual sire or dam and its offspring were compared with all the other unrelated animals. Duncan's test and t-test were used to determine differences ( $P < 0.05$ ).

#### **Results**

Significant differences were not noted between groups in lag time (Table 2). The average lag time over all groups was  $1.27 \pm 0.26$  days with coefficient of variation (CV) 80.32%. In all pairwise comparisons in this study, there were no significant differences in lag times between allotted groups (Table 3).

During the stationary phase, viable cell counts were analyzed (Table 2). The FC group value was significantly higher in SPCC than that for B group ( $P < 0.05$ ). However, there were no significant differences between the three other groups, including FC, MC, and C ( $P > 0.05$ ). The average SPCC over all groups was  $(2.2 \times 10^5 \pm 1.6 \times 10^4 \text{ cells/ml}; 5.34 \pm 0.03 \text{ Log}_{10}\text{cells/ml})$  with a CV of 2.38%. When grouped by age (B and C versus MC and FC), younger groups (MC and FC) showed higher SPCC than older groups (B and C) ( $P < 0.05$ ) (Table 3).

At the second passage, the B and C groups displayed significantly longer MGT ( $0.87 \pm 0.04$  and  $0.90 \pm 0.05$  days, respectively) than the MC and FC groups ( $0.61 \pm 0.06$  and  $0.55 \pm 0.06$  days, respectively) ( $P < 0.05$ ) (Table 2). As depicted in Figure 3, the MGT of the bovine cell lines were influenced by age ( $P = 0.0001$ ). The average MGT over all groups was  $0.72 \pm 0.05$  days. When comparing between groups according to age, significant differences in MGT were noted ( $P < 0.05$ ) (Figures 4 and 5). However, when the comparisons were carried out according to sex, there were no differences in MGT ( $P > 0.05$ ) (Figure 6).



Table 2. Growth parameters of each bovine cell line at passage 2

Group	ID	Lag time (days)	Stationary phase viable cell count (SPCC) (Log <sub>10</sub> cells/ml)	Mean cell generation time (MGT) (days)
<b>Bulls (B)</b>	110*	0.78	5.45	1.38
	1014	3.00	5.05	0.86
	2507	0.00	5.35	0.94
	2505	0.00	5.25	0.80
	Mean±SE	1.00±1.00 <sup>a</sup>	5.22±0.09 <sup>a</sup>	0.87±0.04 <sup>a</sup>
<b>Male Calves (MC)</b>	4020	0.55	5.25	0.72
	4044	0.00	5.50	0.64
	4033	1.00	5.35	0.64
	4090	2.00	5.40	0.45
	Mean±SE	0.89±0.42 <sup>a</sup>	5.38±0.05 <sup>ab</sup>	0.61±0.06 <sup>b</sup>
<b>Cows (C)</b>	6064	1.89	5.30	1.03
	580	0.89	5.40	0.86
	670	1.89	5.30	0.95
	1027	0.78	5.25	0.78
	Mean±SE	1.36±0.31 <sup>a</sup>	5.31±0.03 <sup>ab</sup>	0.90±0.05 <sup>a</sup>
<b>Female Calves (FC)</b>	4035	1.00	5.50	0.60
	4076	2.00	5.55	0.38
	4075	3.22	5.25	0.66
	4060	0.89	5.40	0.55
	Mean±SE	1.78±0.54 <sup>a</sup>	5.43±0.07 <sup>b</sup>	0.55±0.06 <sup>b</sup>
<b>Total</b> Mean±SE		1.27±0.26	5.34±0.03	0.72±0.05
C.V. (%)**		80.32	2.38	25.96

Data were generated by the equation (Dawes, 1980) in conjunction with plots for the logarithmic phases of growth.

\*These bull data were excluded from overall statistics. The initial seeding ( $5 \times 10^4$  cells/ml) of this animal for passage 2 was different from other treatments ( $1 \times 10^4$  cells/ml).

\*\*Coefficient of variation (%) = (standard deviation x100)/mean.

<sup>a,b</sup>Different superscripts within the same column indicate significant differences ( $P < 0.05$ ).

Table 3. Pairwise comparisons of growth parameters of bovine cell lines at passage 2

Comparison	Group	No. of animals	Lag time (days)	Stationary phase viable cell count (Log <sub>10</sub> cells/ml)
Age	B and C	7	1.21±0.42 <sup>a</sup>	5.27±0.04 <sup>a</sup>
	MC and FC	8	1.33±0.36 <sup>a</sup>	5.40±0.04 <sup>b</sup>
Age 1	B	3	1.00±1.00 <sup>a</sup>	5.22±0.09 <sup>a</sup>
	MC	4	0.89±0.42 <sup>a</sup>	5.38±0.05 <sup>a</sup>
Age 2	C	4	1.36±0.31 <sup>a</sup>	5.31±0.03 <sup>a</sup>
	FC	4	1.78±0.54 <sup>a</sup>	5.43±0.07 <sup>a</sup>
Age/Sex 1	B	3	1.00±1.00 <sup>a</sup>	5.22±0.09 <sup>a</sup>
	FC	4	1.78±0.54 <sup>a</sup>	5.43±0.07 <sup>a</sup>
Age/Sex 2	C	4	1.36±0.31 <sup>a</sup>	5.31±0.03 <sup>a</sup>
	MC	4	0.89±0.42 <sup>a</sup>	5.38±0.05 <sup>a</sup>
Sex	B and MC	7	0.94±0.44 <sup>a</sup>	5.31±0.05 <sup>a</sup>
	C and FC	8	1.57±0.30 <sup>a</sup>	5.37±0.04 <sup>a</sup>
Sex 1	B	3	1.00±1.00 <sup>a</sup>	5.22±0.09 <sup>a</sup>
	C	4	1.36±0.31 <sup>a</sup>	5.31±0.03 <sup>a</sup>
Sex 2	MC	4	0.89±0.42 <sup>a</sup>	5.38±0.05 <sup>a</sup>
	FC	4	1.78±0.54 <sup>a</sup>	5.43±0.07 <sup>a</sup>

B: Bull group, C: Cow group, MC: Male Calf group, FC: Female Calf group. The comparison Age is older versus younger cell lines (B and C versus MC and FC). Age was divided into two subcomparisons by sex including Age 1 and Age 2. Age 1 = older males versus younger males (B versus MC) and Age 2 = older females versus younger females (C versus FC). Age/Sex 1 represents older males versus younger females (B versus FC). Age/Sex 2 means older females versus younger males (C versus MC). Sex refers to males versus females (B and MC versus C and FC). Sex was divided into two sub comparisons by age including Sex 1 and Sex 2. Sex 1 = older males versus older females (B versus C) and Sex 2 = younger males versus younger females (MC versus FC). <sup>a,b</sup>Different superscripts within the same column indicate significant differences (P<0.05).

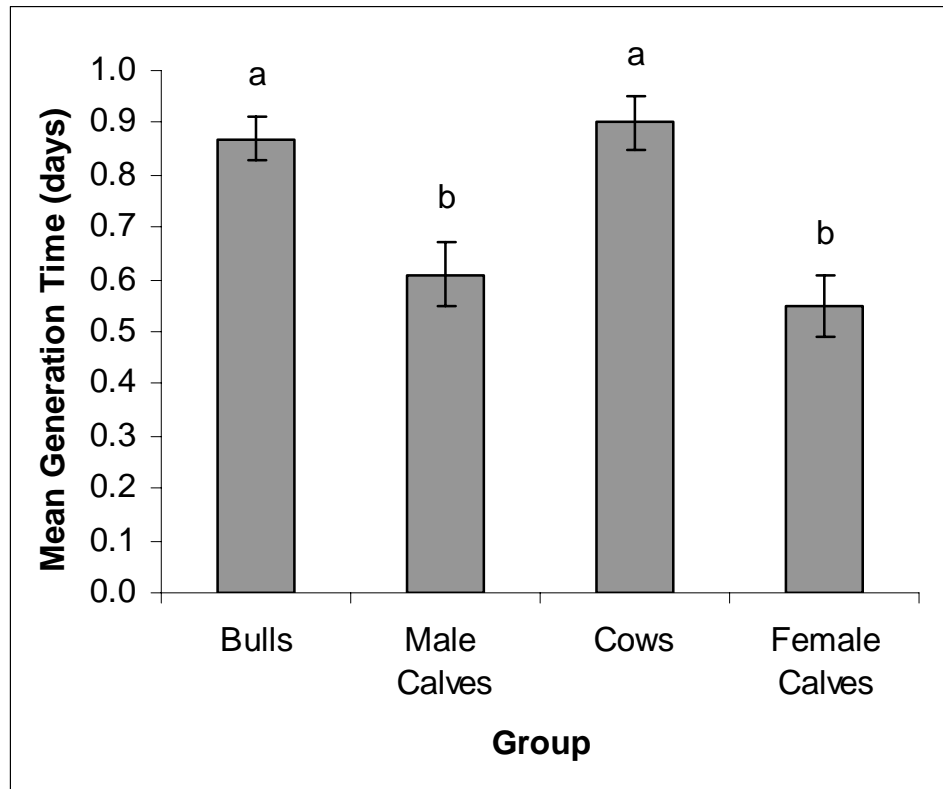


Figure 3. Mean cell generation times for bovine cell lines from each animal group at passage 2. <sup>a,b</sup>Different superscripts indicate significant differences ( $P < 0.05$ ).

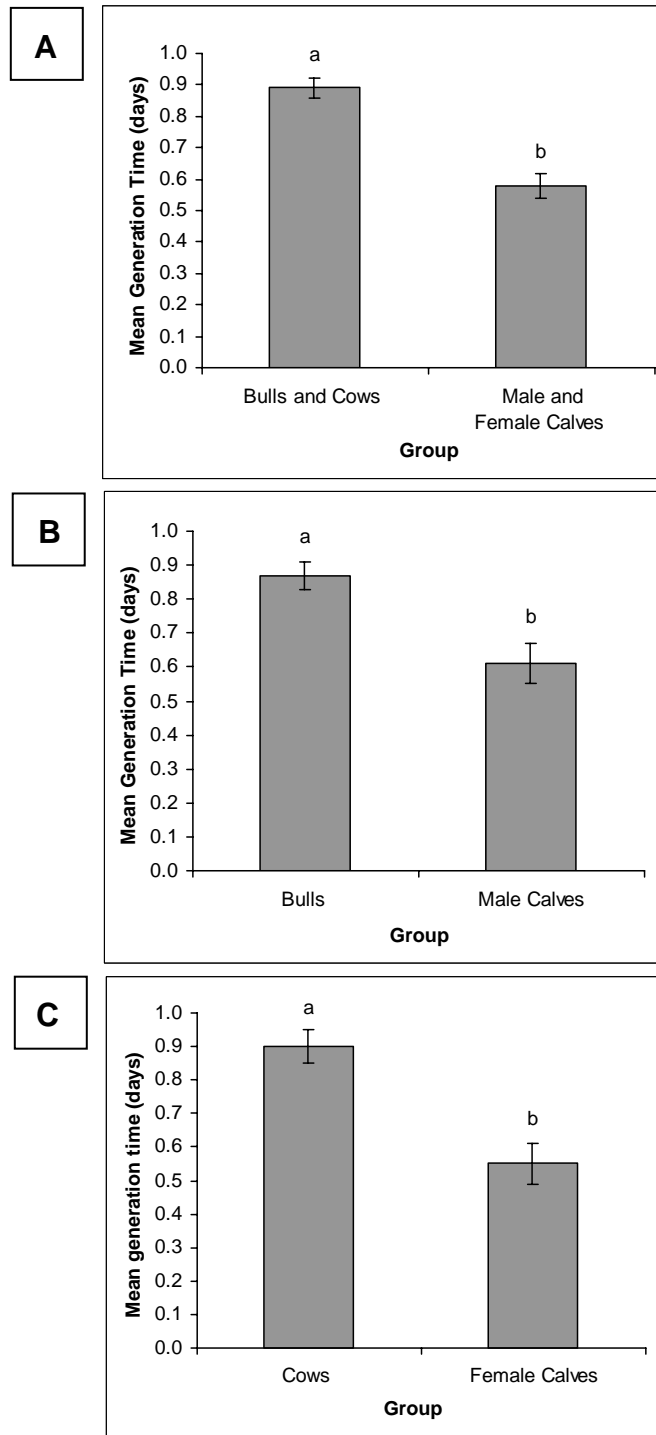


Figure 4. Mean cell generation times for bovine cell lines at passage 2. A: Comparison by Age (older versus younger), Bulls and Cows (n=7) versus Male and Female Calves (n=8). B: Comparison by Age 1 (older males versus younger males), Bulls (n=3) versus Male calves (n=4). C: Comparison by Age 2 (older females versus younger females), Cows (n=4) versus Female Calves (n=4). <sup>a,b</sup>Different superscripts indicate a significant difference (P<0.05).

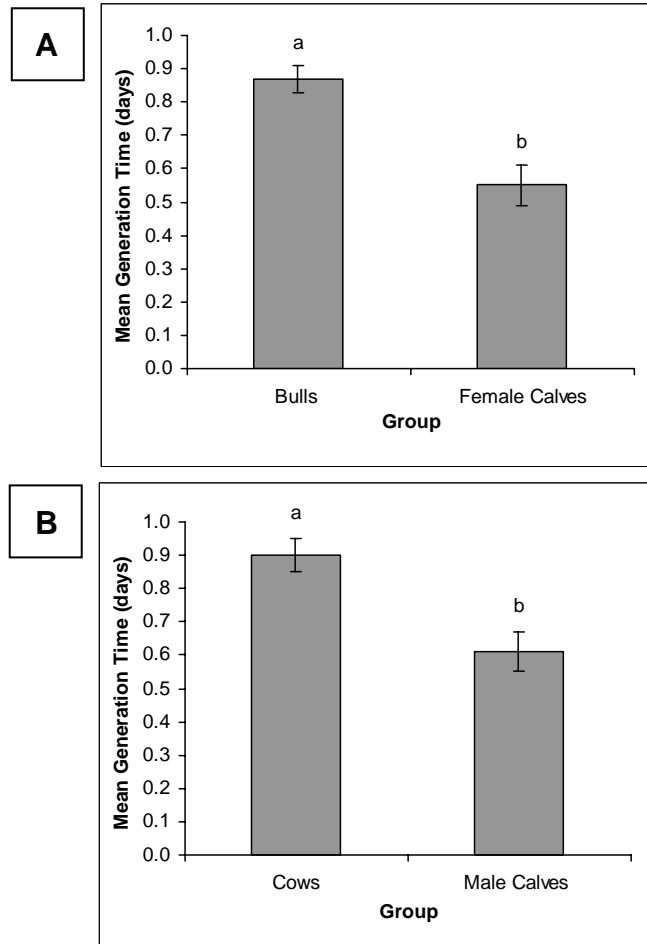


Figure 5. Mean cell generation times for cell lines at passage 2. A: Comparison by Age/Sex 1 (older males versus younger females), Bulls (n=3) versus Female Calves (n=4). B: Comparison by age/sex 2 (older female versus younger male), Cows (n=4) versus Male Calves (n=4). <sup>a,b</sup>Different superscripts indicate a significant difference (P<0.05).

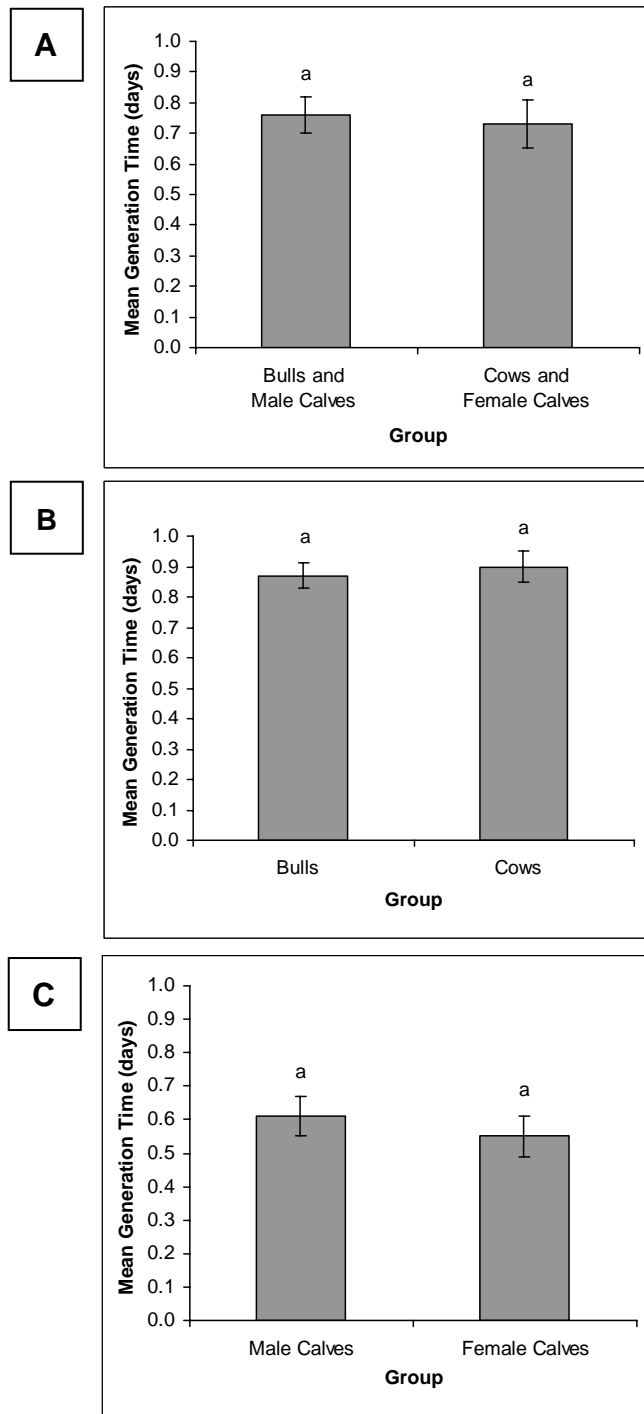


Figure 6. Mean cell generation times (days) for bovine cell lines at passage 2. A: Comparison by sex (males versus females), Bulls and Male Calves (n=7) versus Cows and Female Calves. B: Comparison by sex 1 (older males versus older females), Bulls (n=3) versus Cows (n=4). C: Comparison by sex 2 (younger males versus younger females), Male Calves (n=4) versus Female Calves (n=4). <sup>a,b</sup>Different superscripts column indicate significant difference (P<0.05).

Growth curves of cell lines showed similar patterns by group (Figures 7 and 8). One cell line (#1014 bull) in B group had lower SPCC, and one cell line (female calf #4075) displayed longer lag time in FC group (Figures 7 and 8). Using phase contrast microscopy, the morphology of cells was primarily fibroblast-like, with spindle shapes and with many pseudopodial extensions (Lories *et al.*, 2003) (Figures 9 and 10).

In all but one case, no significant differences ( $P>0.05$ ) were observed for the growth parameters (i.e., lag time, SPCC, and MGT) in the related and unrelated groups (Table 4). A significant difference was found in SPCC between the animals sired by bull #110 and those unrelated to the bull #110 ( $P<0.05$ ). When examining the familial lineage and MGT, each cell line (upper dotted line box) had comparatively slow MGT, or higher rank, than their offspring (lower dotted line box) over all groups (Figure 11) (Table 5). However, there was no relationship between a sire and/or dam and its offspring, whether male or female. Even though both (bull #1014 x cow #6064, bull #1014 x cow #670) had comparatively long MGT over all groups, offspring (male calf #4090, female calf #4075) had a comparatively short MGT over that of the others. There was no clear relationship between sire and dams and their offspring within the group comparisons (Figure 11) (Table 5).

Each cell line maintained more than 98% of cell viability ( $98.64 \pm 0.42$ ,  $98.39 \pm 1.02$ ,  $99.37 \pm 0.22$ , and  $98.96 \pm 0.30\%$  for B, C, MC, and FC, respectively) throughout the growth curves, and no significant differences in cell viability were noted among all four age groups.

## Discussion

At the second passage, Bull and Cow groups have significantly longer MGT than those of other two younger groups (Male Calves and Female Calves) ( $P=0.0001$ ) (Table 2) (Figure 4 A). This indicates that somatic cell growth *in vitro* was influenced by age at passage 2. The fact that the age related comparisons (Figures 4 and 5) showed significant differences in MGT between groups compared ( $P<0.05$ ), an age effect of cells on the MGT was revealed. In contrast, there were no significant differences in sex-related comparisons (Figure 6), which is interpreted to mean that sex was not an influence on the MGT of the bovine cell line ( $P>0.05$ ). These data demonstrated that age but not sex can influence the MGT in bovine (*Bos taurus*) cell line *in vitro* culture at passage 2.

Reports have focused on fibroblast cell line growth patterns along with sera derived from animals of different ages (Carrel and Ebeling, 1921; Baker and Carrel, 1925; Parker, 1931; Kondo and Nomaguchi, 1985; Kondo *et al.*, 1988a, 1988b; Kondo *et al.*, 1989). Previous studies have been inconclusive regarding age-related extracellular serum factors

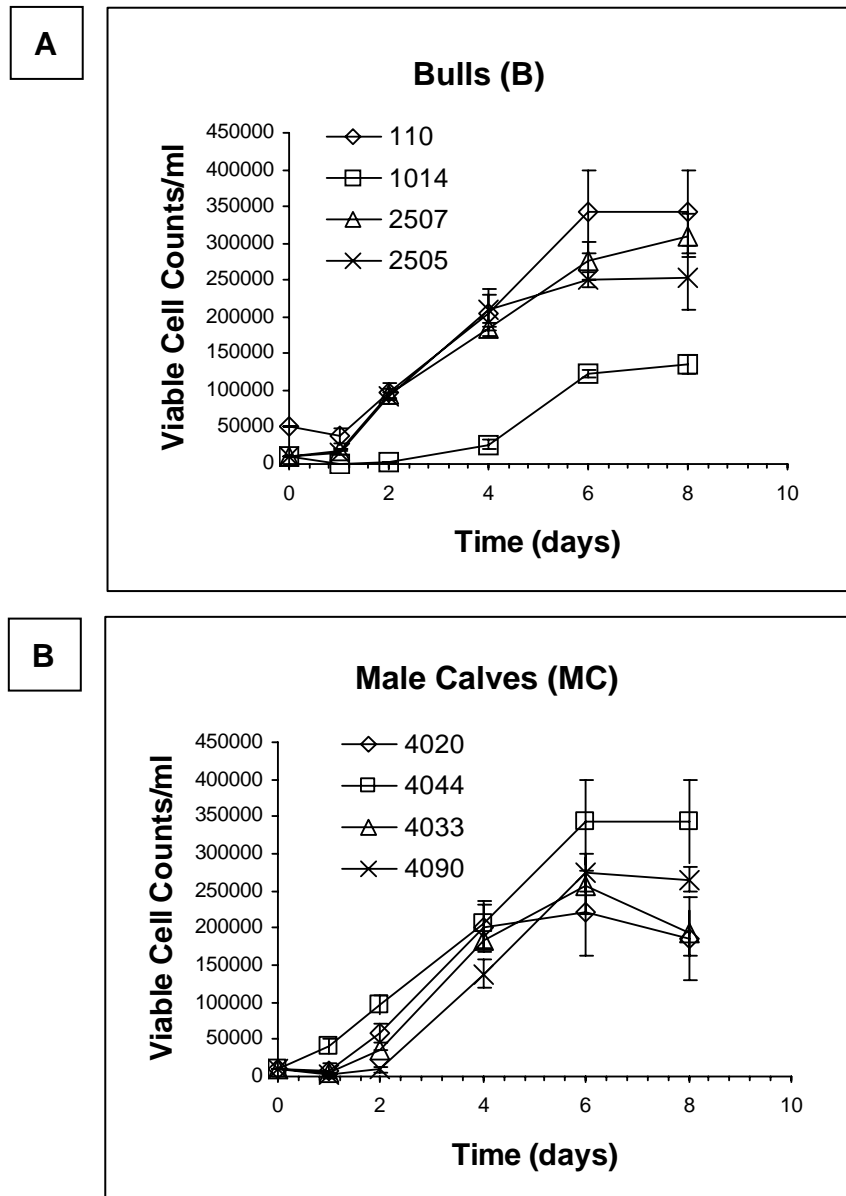


Figure 7. Growth curves of bovine cell lines at passage 2. A: Bull group was composed of male #110, #1014, #2507 and #2505. B: Male Calf group was composed of male #4020, #4044, #4033 and #4090.



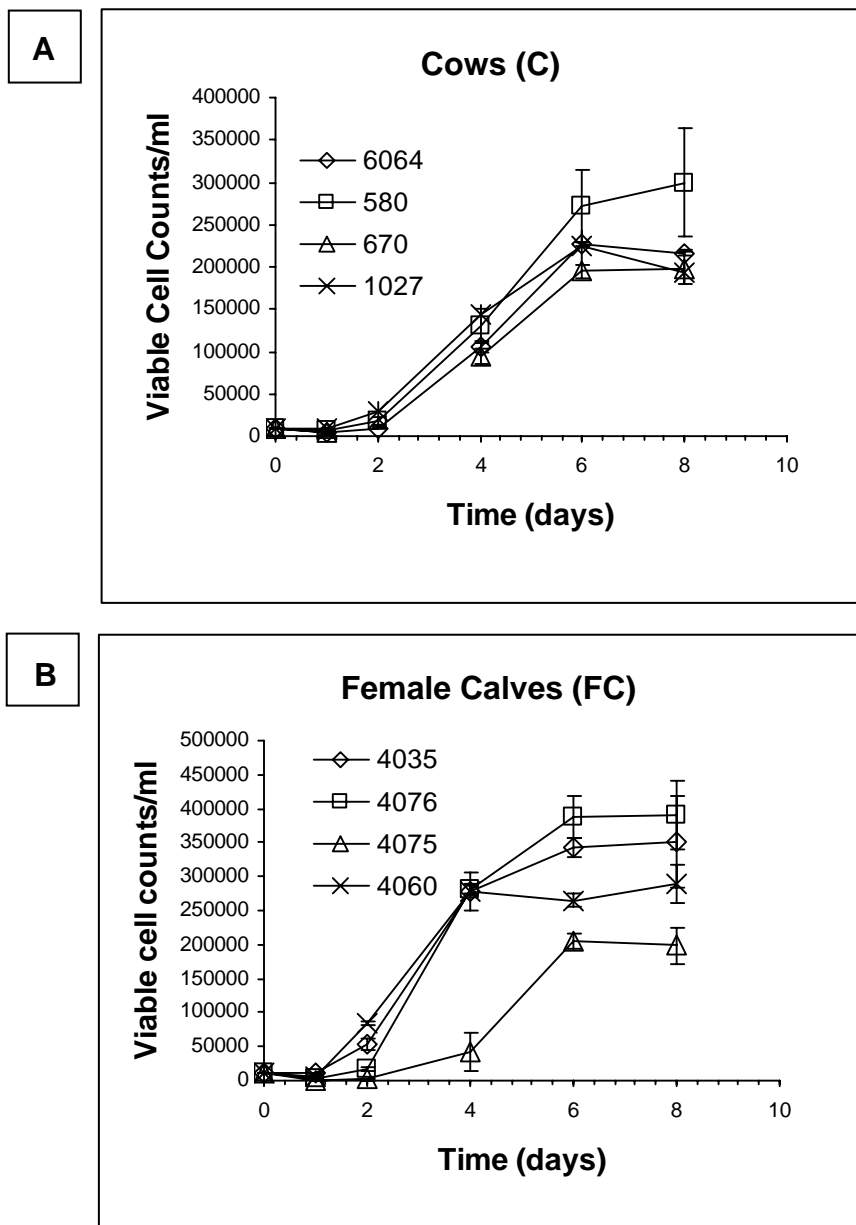


Figure 8. Growth curves of bovine cell lines at passage 2. A: Cow group was composed of female #6064, #580, #670 and #1027. B: Female Calf group was composed of female #4035, #4076, #4075 and #4060.

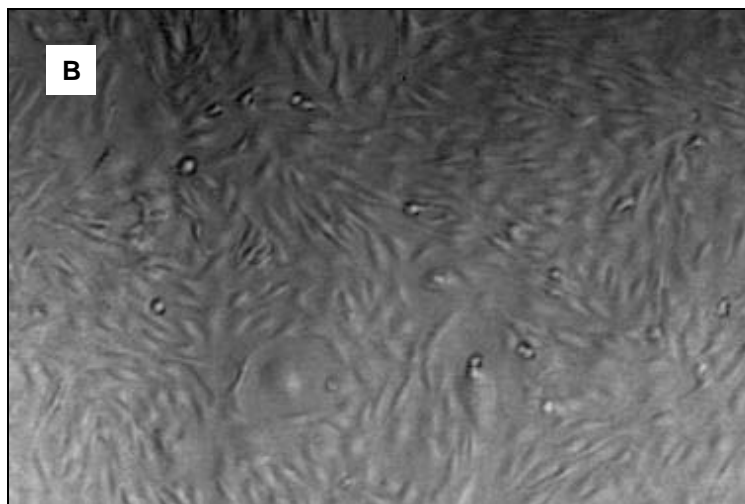
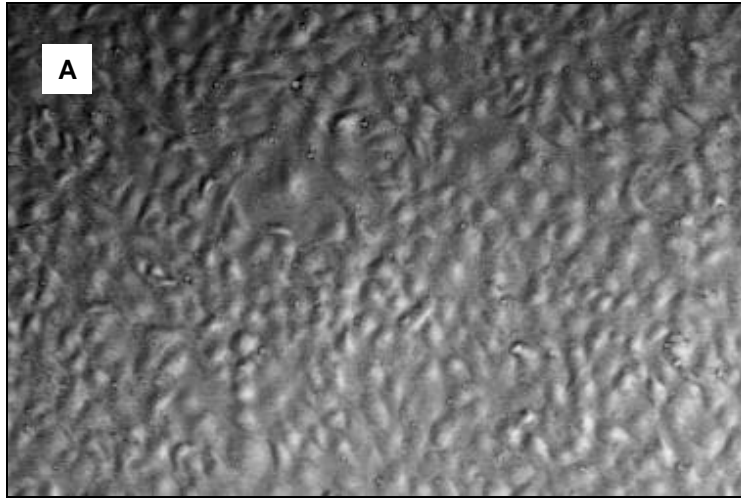


Figure 9. Phase microscopy of bovine cell lines at passage 1 (100X). A: Bull #2505 cell line at compact contact inhibition. B: Cow #6064 cells in a confluent monolayer.

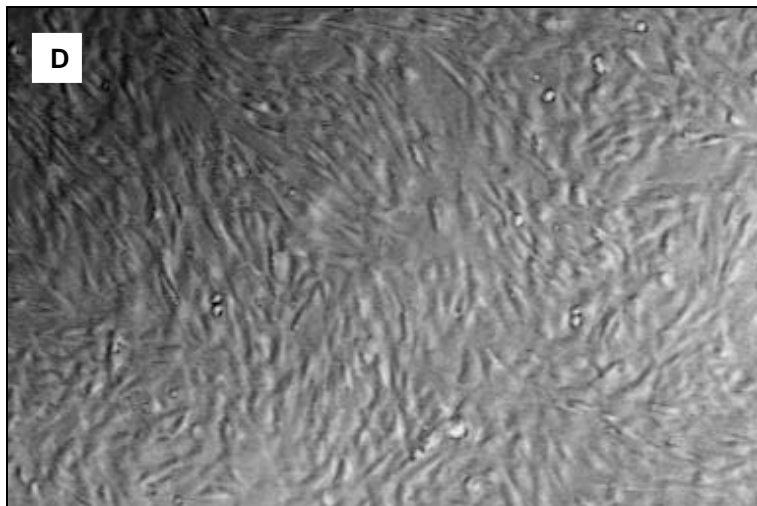
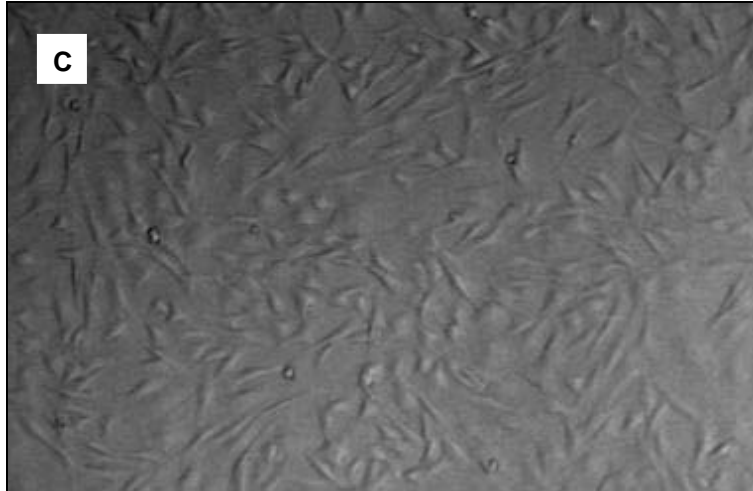


Figure 10. Phase microscopy of bovine cell lines at passage 1 (100X). C: Male calf #4033 cells in a confluent monolayer. D: Female calf #4076 cells at on compact contact inhibition.

Table 4. Growth parameter comparisons between bovine cell lines\* as they relate to familial lineage\*\*

Group	No. of animals	Lag time (days)	Stationary Phase cell count (Log <sub>10</sub> cells/ml)	Mean generation Time (days)
Bull #1014 related	6	1.78±0.47 <sup>a</sup>	5.28±0.05 <sup>a</sup>	0.65±0.06 <sup>a</sup>
Bull #1014 unrelated	9	0.94±0.28 <sup>a</sup>	5.38±0.04 <sup>a</sup>	0.78±0.07 <sup>a</sup>
Bull #110 related	3	1.00±0.58 <sup>a</sup>	5.52±0.02 <sup>a</sup>	0.54±0.08 <sup>a</sup>
Bull #110 unrelated	12	1.34±0.30 <sup>a</sup>	5.30±0.03 <sup>b</sup>	0.77±0.05 <sup>a</sup>
Cow #6064 related	2	1.95±0.06 <sup>a</sup>	5.35±0.05 <sup>a</sup>	0.74±0.29 <sup>a</sup>
Cow #6064 unrelated	13	1.17±0.30 <sup>a</sup>	5.34±0.04 <sup>a</sup>	0.72±0.05 <sup>a</sup>
Cow #670 related	2	2.56±0.67 <sup>a</sup>	5.43±0.13 <sup>a</sup>	0.67±0.29 <sup>a</sup>
Cow #670 unrelated	13	1.08±0.25 <sup>a</sup>	5.35±0.04 <sup>a</sup>	0.71±0.05 <sup>a</sup>

<sup>a,b</sup> Different superscripts within the same column indicate significant differences (P<0.05).

\*Cells at the 2<sup>nd</sup> passage.

\*\*See Figure 2 for familial lineage.

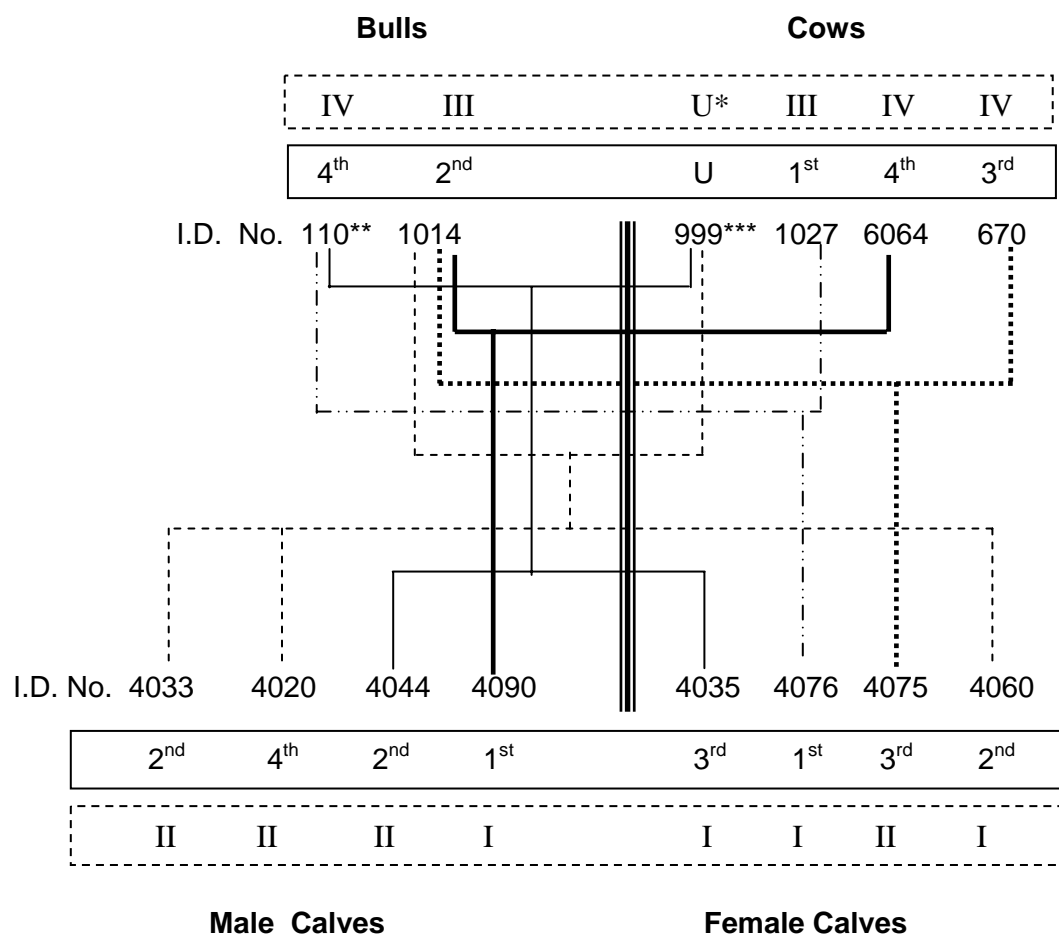


Figure 11. A comparison between bovine familial lineage and cell line mean generation time at passage 2. \*U: Unknown. \*\*This bull's data were excluded from analysis due to different initial seeding ( $5 \times 10^4$  cells/ml) compared with other treatments ( $1 \times 10^4$  cells/ml). \*\*\*999: No data on these cows were obtained on mean cell generation time (MGT). For example, Bull #110 x Cow #999 produced male calf #4044 and female calf #4035.  : Comparison within group. Each individual was ranked from 1<sup>st</sup> to 4<sup>th</sup> within each group, with shorter MGT receiving lower ranks.  : Comparison over all groups. Each individual was ranked from I to IV over all groups. Categories include ranks from I-1<sup>st</sup> to 4<sup>th</sup>, II- 5<sup>th</sup> to 8<sup>th</sup>, III- 9<sup>th</sup> to 12<sup>th</sup>, IV-13<sup>th</sup> to 16<sup>th</sup>.

Table 5. Ranking of mean cell generation time and familial lineage relationship of each bovine cell line at passage 2

Group	ID	Age (Yr.)	Mean generation time (days)	Ranking within each group*	Ranking over all groups*	Ranking over all groups
<b>Bulls (B)</b>	110**	4.25	1.38	4	16	IV
	1014	3.25	0.86	2	11	III
	2507	1.70	0.94	3	13	IV
	2505	1.70	0.80	1	10	III
	Mean±SE	2.73±0.63	0.87±0.04 <sup>a</sup>			
<b>Male Calves (MC)</b>	4020	0.30	0.72	4	8	II
	4044	0.25	0.64	2	5	II
	4033	0.25	0.64	2	5	II
	4090	0.20	0.45	1	2	I
	Mean±SE	0.25±0.02	0.61±0.06 <sup>b</sup>			
<b>Cows (C)</b>	6064	8.50	1.03	4	15	IV
	580	4.25	0.86	2	11	III
	670	4.25	0.95	3	14	IV
	1027	3.30	0.78	1	9	III
	Mean±SE	5.08±1.16	0.90±0.05 <sup>a</sup>			
<b>Female Calves (FC)</b>	4035	0.25	0.60	3	4	I
	4076	0.20	0.38	1	1	I
	4075	0.20	0.66	4	7	II
	4060	0.25	0.55	2	3	I
	Mean±SE	0.23±0.01	0.55±0.06 <sup>b</sup>			

\*A rank from 1<sup>st</sup> to 4<sup>th</sup> was assigned to individuals within each group, with shorter MGT receiving lower ranks. Ranked individuals were assigned to categories, with the first category I containing 1<sup>st</sup> to 4<sup>th</sup> ranks, category II consisting of 5<sup>th</sup> to 8<sup>th</sup> ranks, category III consisting of 9<sup>th</sup> to 12<sup>th</sup>, and category IV consisting of 13<sup>th</sup> to 16<sup>th</sup>. Again shorter MGT were in categories ranked lower (I < IV).

\*\*This bull's data were excluded from analysis due to different initial seeding (5 X 10<sup>4</sup> cells/ml) compared to other treatments (1 X 10<sup>4</sup> cells/ml).

<sup>a,b</sup>Different superscripts within the same column indicate significant difference (P<0.05).

and their impacts on growth of cells in culture. In other words, it has not been conclusive whether sera derived from older subjects had inhibitory effects on cell proliferation or not. It follows that because serum alone is not the sole attribute that inhibits or stimulates cell growth, the age of the cell lines is a parameter to consider in cell proliferation. Reports illustrated that cell lines from younger donors can lead to more active cell proliferation than that in cell lines from older individuals (Nakao *et al.*, 1984; Morris *et al.*, 1990; Kondo and Yonezawa, 1992). In the present study, MGT in cell lines from younger donors (Male Calves and Female Calves) at passage 2 showed significantly shorter MGT than those from older individuals (Bulls and Cows).

This relationship between age and MGT shows similarities with results from wound-healing experiments (Goodson and Hunt, 1979; Grove, 1982; Muggleton-Harris *et al.*, 1982; Cohen *et al.*, 1987; Holt *et al.*, 1992). Those studies consistently reported that younger subjects had faster wound healing results than those of older subjects. Reports on age-related wound healing phenomena illustrate similarities with our study in that cell lines from younger donors could grow faster than those of older subjects.

Various studies have been performed on factors related to sex and their influence on cell proliferation (Faulkner *et al.*, 1989; Kwan *et al.*, 1996; Griffin *et al.*, 2000). Main concerns of those reports were the relationship between sex steroid hormones (estradiol and testosterone) and the differential reactions of cells derived from different sexes of animals. In contrast, with regard to wound repair in conjunction with sex factors, conclusive results are not available (Ashby *et al.*, 1969; Liu *et al.*, 2001; Hawkey *et al.*, 2002). However, it is obvious that in some specific sites of wound, there have been significant difference in wound healing rate along with sex (Liu *et al.*, 2001; Hawkey *et al.*, 2002).

In all these studies involving sex factors, relationships exist between sex-related factors and cell proliferation. These results have been explained by sex hormones inducing endocrinological signals (Evans, 1988) and/or cell characteristics conferred by sex-related gene expression. *In vitro* cell culture systems, as in this study, do not allow for engaging endocrinological signals. This may explain no observed impacts by sex on bovine cell's MGT. Each cell line showed different lag times within its group and over all groups with coefficient of variation of 80.32%. In any other pairwise comparison in this study, lag time was not affected by age or sex (Table 3).

During the stationary phase, the viable cell counts of the Female Calf group showed ( $P < 0.05$ ) higher numbers than the Bull group (Table 2). Interestingly, younger groups (Male

Calves and Female Calves) showed higher stationary phase viable cell counts (SPCC) than older groups (Bulls and Cows) ( $P < 0.05$ ), indicating age-related factors could influence the SPCC (Table 3).

In this study, cell cycle synchronization was attempted by contact inhibition (Holley and Kiernan, 1968; Stoker, 1973). According to the pattern of each growth curve including lag time, SPCC and MGT, cell cycle synchronization seemed not to be achieved completely (Table 2) (Figures 7 and 8). This incomplete synchronization support reports that the release of arrested cells from inhibition can not produce cell lines acting like cells during normal division cycle (Di Matteo *et al.*, 1995; Cooper, 1998; Cooper, 2003). Generally passage 2 is considered an early time to achieve homogeneous fibroblast cell lines (Zimmermann *et al.*, 2001; Pap *et al.*, 2003), if more a homogeneous cell line is established thus, there appears to be a possibility for a more synchronized pattern of cell lines.

Evaluating familial lineage in this study, the comparison between related groups and unrelated groups showed that most comparisons do not show significant differences in all parameters including lag time, SPCC, and MGT (Table 4). In contrast, the SPCC, statistically bull #110-related group illustrated difference compared to that of cow #110-unrelated group. With more cell lines related to bull #110 by familial lineage history, SPCC investigation is needed to confirm this.

Calves did not always follow the MGT phenotype of parents (Figure 11) (Table 5). There was no evidence that either dam or sire can affect the MGT of offspring (Figure 11) (Table 5). In a report by Clark *et al.* (2003), the proliferative lifespan of ovine cell lines, which was determined by growing the cell lines continuously until senescence, showed innate and genetically determined proliferation capacity (Mueller *et al.*, 1980; Clark *et al.*, 2003). In the study by Muller *et al.* (1980), cultures of bovine fetal aortic endothelial cells showed senescence after 80 cumulative population doublings. The growth rates in the logarithmic phase decreased as the population doubling level increased. An important point from the experiment by Clark *et al.* (2003) is that up to 60 days after *in vitro* ovine fibroblasts culture there was not so conspicuous difference in growth rate (or MGT) among cell lines. However, after 60 days of *in vitro* culture, there were conspicuous cell growth rate change (extension of doubling time) and this eventually drove the senescence of each cell line. If it is the case, a possible explanation could be that specific telomere controlling genes, i.e., genes encoding telomeric repeat binding factor (TRF) and/or telomere reverse transcriptase (TERT) (Moyzis *et al.*, 1988; Hastie *et al.*, 1990; Harley *et al.*, 1990; Kim *et al.*, 1994; Bianchi *et al.*, 1997; Cui



*et al.*, 2003; Karlseder *et al.*, 2003) are related to MGT and proliferative lifespan of each mammalian cell line and those genes can only be appeared to work effectively at later passage (60 days after *in vitro* culture). Because at early passage there should be not so much difference in telomere length even though the telomere resistant genes had worked along with aging (passages progression).

Each cell line showed high viability throughout the growth curves (98 to 99%), which indicates that stable cell maintenance and proper cell harvest was conducted in this study. It is possible to conclude that the viability of early passage (passage 2) bovine cell line was stable.

In conclusion, at passage 2, the bovine cell line was influenced not by sex but by age in terms of MGT. The younger animal group (Male Calves and Female Calves) showed shorter MGT than the older group (Bulls and Cows). This pattern showed similarities to studies on sera effect derived from animals of different ages and differences in wound healing between young and older cell lines. There was no obvious relationship between sires and dams and their offspring in terms of MGT. Rather than individual genetics, age had more effect on cell growth patterns (specifically with MGT) of bovine cell lines at early passage (passage 2).

## CHAPTER IV

### THE EFFECT OF DONOR ANIMAL AGE AND SEX ON CELL GROWTH PARAMETERS AND CELL CYCLE PHASES OF FROZEN-THAWED BOVINE CELL LINES

#### Introduction

The somatic cell nuclear transfer (NT) technique has been used with various mammalian species (Cibelli *et al.*, 1998; Wakayama *et al.*, 1998; Baguisi *et al.*, 1999; Betthauser *et al.*, 2000; Shin *et al.*, 2002), however, the overall efficiency of NT is less than 1% of reconstructed embryos (Han *et al.*, 2003). To overcome this low NT efficiency, tetraploid complementation (Nagy *et al.*, 1990; Nagy *et al.*, 1993; Guillemot *et al.*, 1994; Duncan *et al.*, 1997; Iwasaki *et al.*, 2000; Misra *et al.*, 2001; Eggan *et al.*, 2002) or inner cell mass (ICM) transplantation (Polzin *et al.*, 1987; Anderson, 1988; MacLaren *et al.*, 1992; Lasley *et al.*, 1994; Rorie *et al.*, 1994) have been attempted.

The DNA cell cycle phase of donor nuclei has been of primary importance to the success rate of NT and embryo reconstruction (Wang, 1991; Kitagawa *et al.*, 1994; Wilmut *et al.*, 1997; Campbell *et al.*, 1996; Cooper, 2003). In the first production of a live offspring from somatic cells, donor cells were arrested in the quiescent G<sub>0</sub> stage (Wilmut *et al.*, 1997). Though some somatic cell cloning have been achieved with donor cells in G<sub>1</sub> and G<sub>2</sub>/M phase (Cibelli *et al.*, 1998; Ono *et al.*, 2001), better efficiencies have been obtained using nuclei in the G<sub>0</sub>/G<sub>1</sub> phase. To synchronize growth in G<sub>0</sub>/G<sub>1</sub> phase, several synchronization methods are available, such as serum starvation (Kues *et al.*, 2000; Peura, 2001), contact inhibition (Holley and Kiernan, 1968; Stoker, 1973; Gómez *et al.*, 2003) and the reversible cycle inhibitor (roscovitine) (Boquest *et al.*, 1999; Gibbons *et al.*, 2001).

Results vary regarding the effects of sera derived from animals of different ages on mammalian cell cultures (Carrel and Ebeling, 1921; Baker and Carrel, 1925; Parker, 1931; Kondo and Nomaguchi, 1985; Kondo *et al.*, 1988a, 1988b). Some reports illustrated that animal age can negatively affect cell proliferation (Carrel and Ebeling, 1921; Baker and Carrel, 1925; Parker, 1931; Kondo *et al.*, 1989), yet others showed no relationship between animal age and effect on cell proliferation (Kondo and Nomaguchi, 1985; Kondo *et al.*, 1988a, 1988b). The potential impact of sex on cell development in culture has focused on sex steroid hormones, including estradiol and testosterone (Haug *et al.*, 1976; Kaipia *et al.*, 1994; Kwan *et al.*, 1996; Moraghan *et al.*, 1996; Mossuz *et al.*, 1998; Medina *et al.*, 2000; Griffin *et al.*, 2000; Ormerod *et al.*, 2003).

Bovine fetal fibroblasts, which are often used in NT, have 30 to 50 population doublings before senescence occurs (Polejaeva and Campbell, 2000). There have been two points of view about the population doubling capacities. The first is that the population doubling capacity can be mainly adjusted by environmental differences. Schneider and Mitsui (1976) showed that the onset of cell senescence *in vitro* culture of human skin fibroblast cells derived from old (63 to 92 years) individuals was faster than that of young donor's (21 to 36 years). Nichols *et al.* (1977) found that human diploid fibroblast-like cell strain IMR-90 derived from one female embryo showed three different doubling capacities according to conditions including seeding density, growth medium, medium volume and subcultivation reagents. Smith and Whitney reported (1980) that even the two human fibroblast cells derived from a single mitosis showed different population doubling capacity.

In contrast, there have been reports stressing the importance of inherited genetic characteristics. Cristofalo *et al.* (1998) found no relationship between donor age and replicative lifespan (doubling capacity) in human fibroblasts in *in vitro* culture. Clark *et al.* (2003) reported that the cell doubling capacity could vary widely between different cell lines and thus, genetics may have an important role in determining this capacity, which is important when selecting a cell line for NT (Clark *et al.*, 2003).

In this study, the influence of the sex and age of the donor animal on the frozen thawed bovine cell line growth pattern and the DNA synchronization efficiency were investigated. The hypothesis is that either sex or age of donor animal from which somatic cells were derived does influence the pattern of cell growth patterns. The objective of this study was to investigate growth patterns of frozen-thawed Angus (*Bos taurus*) cell lines at passage 4, derived from animals of different sex or age. Such data could be used as a standard for bovine donor tissue and then subsequently for cell manipulation for NT.

## **Materials and Methods**

### **Cryopreservation of Cell Lines**

Confluent monolayers from each cell line (Table 1) at passage 2 were cryopreserved. Each trypsinized and resuspended cell line was centrifuged at 400 x g for 3 minutes, and the pellet was resuspended with freezing medium of 10% dimethyl sulfoxide (DMSO) (Sigma Chemical Company St. Louis, MO), 10% FBS (Hyclone Laboratories, Logan, UT) and 50 µg/ml of gentamycin (Gibco, St. Louis, MO) in Dulbecco's modified eagle medium (DMEM) and cooled to -80°C (at 1°C per minute) in Mr. Frosty (Nalgene, Rochester,

NY) filled with methanol for 24 hours (Kątska *et al.*, 2002). Frozen cells in vials were transferred to a liquid nitrogen tank at -196 °C.

After 2 months, these cell lines were thawed in 38°C water for 45 seconds, and transferred immediately with 4 ml of M1DMEM into 15-ml centrifuge tubes (Corning, New York, NY). The M1DMEM was composed of DMEM (Gibco, Grand Island, NY) with 10% FBS (Hyclone Laboratories, Logan, UT), 50 µg/ml of kanamycin (Sigma, Grand Island, NY) and 50 µg/ml of gentamycin (Gibco, St. Louis, MO). After 5 minutes of centrifugation at 400 x g, the pellet was resuspended with M1DMEM and plated on 35 x 10 mm Falcon<sup>FM</sup> plastic Petri dishes. For the initial seedings, 2 ml at  $5 \times 10^5$  cells/ml were used for passage 3. The 4<sup>th</sup> passage for each of the 16 cell lines were used in this experiment. For the 4<sup>th</sup> passage, 1 ml was seeded at  $1 \times 10^4$  cells/ml in M1DMEM in 1 well of a 4-well dish (Nunc, Roskilde, Denmark). The surface area of 1 well of a 4-well plate was 1.13 cm<sup>2</sup>.

#### Maintenance of Cell Lines

Cell lines were incubated in 5% CO<sub>2</sub> in air at 38°C (Series II water jacketed CO<sub>2</sub> incubator, Thermoforma, Marietta, OH). Medium was changed every three days with M1DMEM. When the culture of the passage 3 was reached 100% confluency, then 3 to 4 days at contact inhibition was maintained to induce cell cycle synchronization.

#### Growth Curve

On days 1, 2, 4, 6 and 8, the cells were obtained after washing with DMEM and trypsinization with 700 µl of trypsin-EDTA (Sigma) for 5 minutes in 5% CO<sub>2</sub> at 38°C. The trypsinized cells were transferred to a 1.5-ml microfuge tube and centrifuged at 400 x g for 4 minutes. Viable cell counts in each sample in duplicate were made using 0.4% trypan blue and a hemocytometer (Cambridge, Buffalo, NY). The remaining cell suspensions were used for flow cytometry studies of the cell cycle.

#### Mean Generation Time and Other Growth Parameters

The mean cell generation time (MGT) of each cell line was calculated using an equation in conjunction with plots depicting logarithmic phases of growth (Dawes, 1980) (Figure 1). The interval (lag time) from the start of cell seeding into culture to the start of their logarithmic phase of growth was recorded (Table 2). The viable cell counts obtained during stationary phase, considered the stationary phase viable cell counts (SPCC), were recorded (Table 2). These parameters have been used in the quantitative evaluation of cell growth patterns (Mangels *et al.*, 1978; Hutson *et al.*, 1988) and then were recorded for each cell line in this study.

### Cell Size Distribution

To place cells into two size categories, a hemocytometer, having a grid of known distance between lines, was used with 10 cells selected randomly from each cell line. Cells were categorized as greater or less than 25  $\mu\text{m}$  in their widest cell diameter. Data were obtained during passage 4 on days 2, 4, 6 and 8 and scored as “1” or “2”, with a score of “1” being less than 25  $\mu\text{m}$  and “2” being greater than 25  $\mu\text{m}$ .

### Flow Cytometry

For cell cycle analysis, a modification of a fixation procedure was performed (Aggarwal and Gupta, 1998). The DNA cycle of each sample in duplicate was analyzed on day 0. Duplicates were made for each sample. The cell suspensions were centrifuged at 400 x g for 5 minutes in 1.5-ml microfuge tubes, the supernatant was discarded, and the pellets were resuspended in 1 ml of 20% cold ( $-20^{\circ}\text{C}$ ) methanol, and stored ( $4^{\circ}\text{C}$ ) for 24 to 72 hours. The fixed cells were centrifuged at 400 x g for 5 minutes and the pellet was resuspended and washed twice with Hanks' Balanced Salts Solution (HBSS) (Sigma, St. Louis, MO). Cells were stained with equal volumes of 0.11% sodium citrate containing 50  $\mu\text{g}/\text{ml}$  propidium iodide (PI), 100  $\mu\text{g}/\text{ml}$  of RNase and 0.1% (v/v) Triton X-100 for 20 minutes at  $38^{\circ}\text{C}$  in the dark. Each cell line was analyzed with a flow cytometer (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA [BDIS]), and 10,000 events were collected with FL2A linear, using linear size and scatter parameters and FL2 doublet discrimination mode. Histograms and dot plots were constructed using Cell Quest software (BDIS). Percentages of cells in three different phases ( $G_0/G_1$ , S, and  $G_2/M$ ) of the cell cycle were calculated using ModFit LT software (Verity Software House, Topsham, ME).

### Familial Lineage and Mean Generation Time

Same methods were used as described in the Material and Methods section of Chapter III (Figure 2).

### Statistical Analysis

Data on MGT, lag time and SPCC were analyzed by ANOVA, with sex, age and their interactions in the model. Then, each group was compared with another group in a 2 x 2 factorial arrangement. For a pairwise analysis (Table 7), eight pairs of comparisons were analyzed. Two-way ANOVA was used to analyze the data on cell cycle comparisons (Table 9). Total comparison and two pairwise comparisons were considered including categorization by age or by sex. The Tukey multiple comparisons were used to determine differences. Values are reported as mean  $\pm$  standard error of the mean.

For the comparison of related animal effect, individual sire and dam mating and subsequent offspring were compared with all the other unrelated animals (Table 10). Duncan's test and t-test were used to determine differences.

## Results

At the fourth passage, there were no differences among four groups in lag time (Table 6). The average lag time over all groups was  $0.70 \pm 0.13$  days with the coefficient of variation (CV) 76.86%. In all pairwise comparisons (Table 7), there were no significant differences in lag time between groups ( $P > 0.05$ ).

There were no significant differences in mean cell generation time (MGT) of cell lines among four groups ( $P > 0.05$ ) with an average  $0.84 \pm 0.05$  days and with CV = 24.9% (Table 6). In all pairwise comparisons (Table 7), there were no significant differences in MGT ( $P > 0.05$ ). Therefore, at passage 4, the MGT of frozen-thawed bovine cell lines were not influenced by either age or sex ( $P > 0.05$ ).

The stationary phase viable cell counts (SPCC) of FC group showed the highest number followed by C, MC, and B group (Table 6). However, there were no significant differences among groups ( $P > 0.05$ ) with the coefficient of variation (CV) 3.81%. In pairwise comparisons, when all groups were categorized by sex (Figure 12), male groups (B and MC) illustrated lower SPCC than female groups (C and FC) ( $P < 0.05$ ). The SPCC of the male group ( $1.1 \times 10^5 \pm 1.3 \times 10^4$  cells /ml;  $5.05 \pm 0.05$  Log<sub>10</sub>cells /ml) was significantly lower ( $P < 0.05$ ;  $P = 0.04$ ) from that of female group ( $1.7 \times 10^5 \pm 3.0 \times 10^4$  cells/ml;  $5.24 \pm 0.07$  Log<sub>10</sub>cells/ml) (Figure 12). When the sex effect was considered, this pattern was consistent (Figures 12 and 13) with higher numerical value for SPCC in both female groups. In all comparisons factoring in age, no significant differences were found among groups compared ( $P > 0.05$ ) in this study (Figure 14).

As shown in Figure 15, Female Calves group maintained small-size cells throughout the growth period thereby, exhibiting a different size patterns from those of Bull, Cow and Male Calf groups. At the second half of the growth period, cells from females were significantly smaller than cells from males ( $P < 0.05$ ) (Table 8).

In G<sub>0</sub>/G<sub>1</sub> phase analysis, on day 0 at passage 4, Female Calves and Male Calves showed higher percentage of G<sub>0</sub>/G<sub>1</sub> phase than that of Cows (Figures 16 and 17, Table 9). In G<sub>2</sub>/M phase analysis, Bulls showed higher percentage of G<sub>2</sub>/M phase than that of group Male Calves and Female Calves ( $P < 0.05$ ). There were no significant differences among the

Table 6. Growth parameters of each bovine cell line at passage 4

Group	ID	Lag time (days)	Stationary phase viable cell count (SPCC) (Log <sub>10</sub> cells/ml)	Mean generation Time (MGT) (days)
<b>Bulls (B)</b>	110	0.71	5.11	0.74
	1014	0.94	4.74	1.25
	2507	0.94	5.00	0.81
	2505	0.71	5.21	0.73
	Mean±SE	0.83±0.07 <sup>a</sup>	5.02±0.10 <sup>a</sup>	0.88±0.12 <sup>a</sup>
<b>Male Calves (MC)</b>	4020	0.71	5.11	0.80
	4044	0.94	5.00	0.85
	4033	1.53	5.21	0.61
	4090	0.00	5.00	1.20
	Mean±SE	0.80±0.32 <sup>a</sup>	5.08±0.05 <sup>a</sup>	0.87±0.12 <sup>a</sup>
<b>Cows (C)</b>	6064	0.00	5.32	0.92
	580	1.18	4.84	1.22
	670	1.41	5.16	0.67
	1027	0.00	5.42	0.77
	Mean±SE	0.65±0.38 <sup>a</sup>	5.19±0.13 <sup>a</sup>	0.89±0.12 <sup>a</sup>
<b>Female Calves (FC)</b>	4035	0.00	5.16	0.76
	4076	0.00	5.37	0.91
	4075	0.94	5.26	0.70
	4060	1.18	5.42	0.57
	Mean±SE	0.53±0.31 <sup>a</sup>	5.30±0.06 <sup>a</sup>	0.74±0.07 <sup>a</sup>
<b>Total</b> Mean±SE		0.70±0.13	5.15±0.05	0.84±0.05
C.V.(%)*		76.86	3.81	24.90

Data were generated by the equation in conjunction with plots depicting logarithmic phases of growth (Dawes, 1980) (Figure 1).

\*Coefficient of variation (%) = (standard deviation x 100)/mean.

<sup>a,b</sup>Different superscripts within the same column indicate significant differences (P<0.05).

Table 7. Pairwise comparisons of growth parameters of bovine cell lines at passage 4

Comparison	Group	No. of animals	Lag time (days)	Mean cell generation time (MGT) (days)
Sex	B and MC	8	0.81±0.15 <sup>a</sup>	0.87±0.08 <sup>a</sup>
	C and FC	8	0.59±0.22 <sup>a</sup>	0.82±0.07 <sup>a</sup>
Sex 1	B	4	0.83±0.07 <sup>a</sup>	0.88±0.12 <sup>a</sup>
	C	4	0.65±0.38 <sup>a</sup>	0.89±0.12 <sup>a</sup>
Sex 2	MC	4	0.80±0.32 <sup>a</sup>	0.87±0.12 <sup>a</sup>
	FC	4	0.53±0.31 <sup>a</sup>	0.74±0.07 <sup>a</sup>
Age/sex 1	B	4	0.83±0.07 <sup>a</sup>	0.88±0.12 <sup>a</sup>
	FC	4	0.53±0.31 <sup>a</sup>	0.74±0.07 <sup>a</sup>
Age/sex 2	C	4	0.65±0.38 <sup>a</sup>	0.89±0.12 <sup>a</sup>
	MC	4	0.80±0.32 <sup>a</sup>	0.87±0.12 <sup>a</sup>
Age	B and C	8	0.74±0.18 <sup>a</sup>	0.89±0.08 <sup>a</sup>
	MC and FC	8	0.66±0.21 <sup>a</sup>	0.80±0.07 <sup>a</sup>
Age 1	B	4	0.83±0.07 <sup>a</sup>	0.88±0.12 <sup>a</sup>
	MC	4	0.80±0.32 <sup>a</sup>	0.87±0.12 <sup>a</sup>
Age 2	C	4	0.65±0.38 <sup>a</sup>	0.89±0.12 <sup>a</sup>
	FC	4	0.53±0.31 <sup>a</sup>	0.74±0.07 <sup>a</sup>

B: Bull group, C: Cow group, MC: Male Calf group, FC: Female Calf group. The comparison Age means older versus younger cell lines (B and C versus MC and FC). Age was divided into two subcomparisons by sex including Age 1 and Age 2. Age 1 is older males versus younger males (B versus MC) and Age 2 is older females versus younger females (C versus FC). Age/sex 1 = older males versus younger females (B versus FC). Age/sex 2 = older females versus younger males (C versus MC). Sex refers to male versus female (B and MC versus C and FC). Sex was divided into two sub comparisons by age including Sex 1 and Sex 2. Sex 1 means older males versus older females (B versus C) and Sex 2 refers to younger males versus younger females (MC versus FC). <sup>a,b</sup>Different superscripts within the same column indicate significant differences (P<0.05).



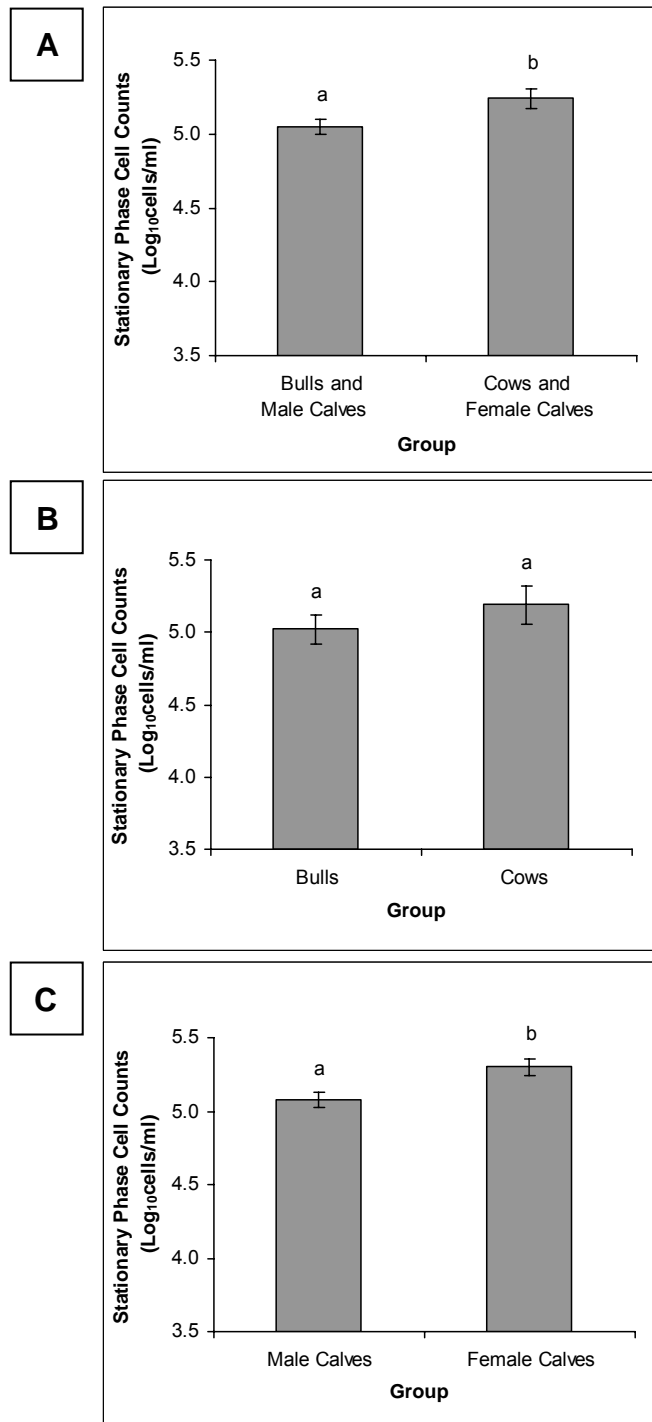


Figure 12. Stationary phase viable cell counts for bovine cell lines at passage 4. A: Comparison by Sex (male versus female), Bulls and Male Calves (n=8) versus Cows and Female Calves. B: Comparison by Sex 1 (older males versus older females), Bulls (n=4) versus Cows (n=4). C: Comparison by Sex 2 (youner males versus younger females), Male Calves (n=4) versus Female Calves (n=4). <sup>a,b</sup>Different superscripts indicate significant difference (P<0.05).

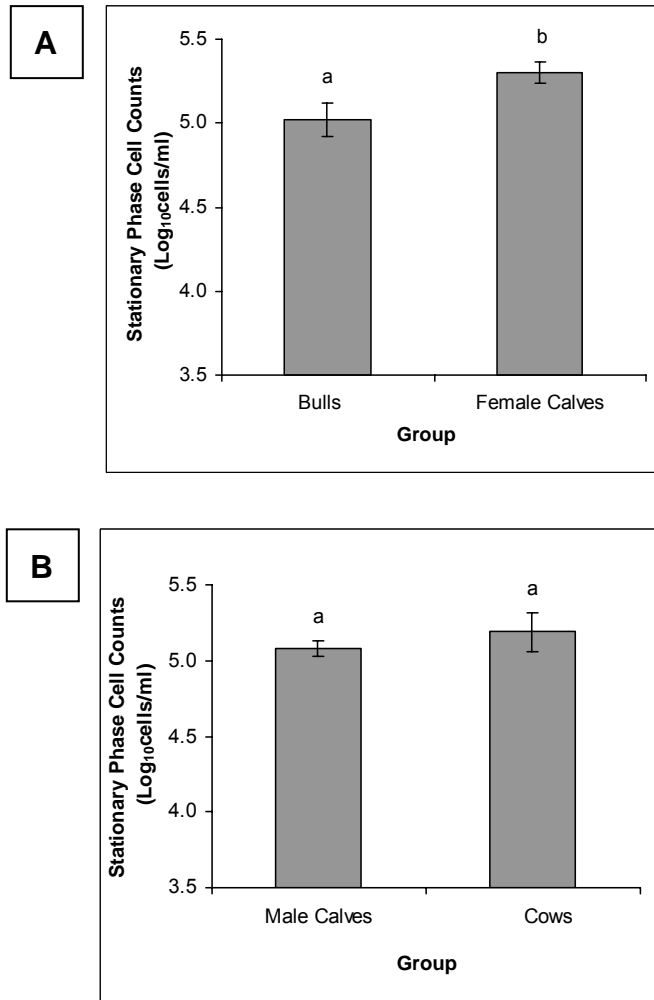


Figure 13. Stationary phase viable cell counts for bovine cell lines at passage 4. A: Comparison by Age/sex 1 (older males versus younger females), Bulls (n=4) versus Female Calves (n=4). B: Comparison by Age/Sex 2 (older females versus younger males), Cows (n=4) versus Male Calves (n=4).<sup>a,b</sup>Different superscripts indicate significant difference ( $P < 0.05$ ).

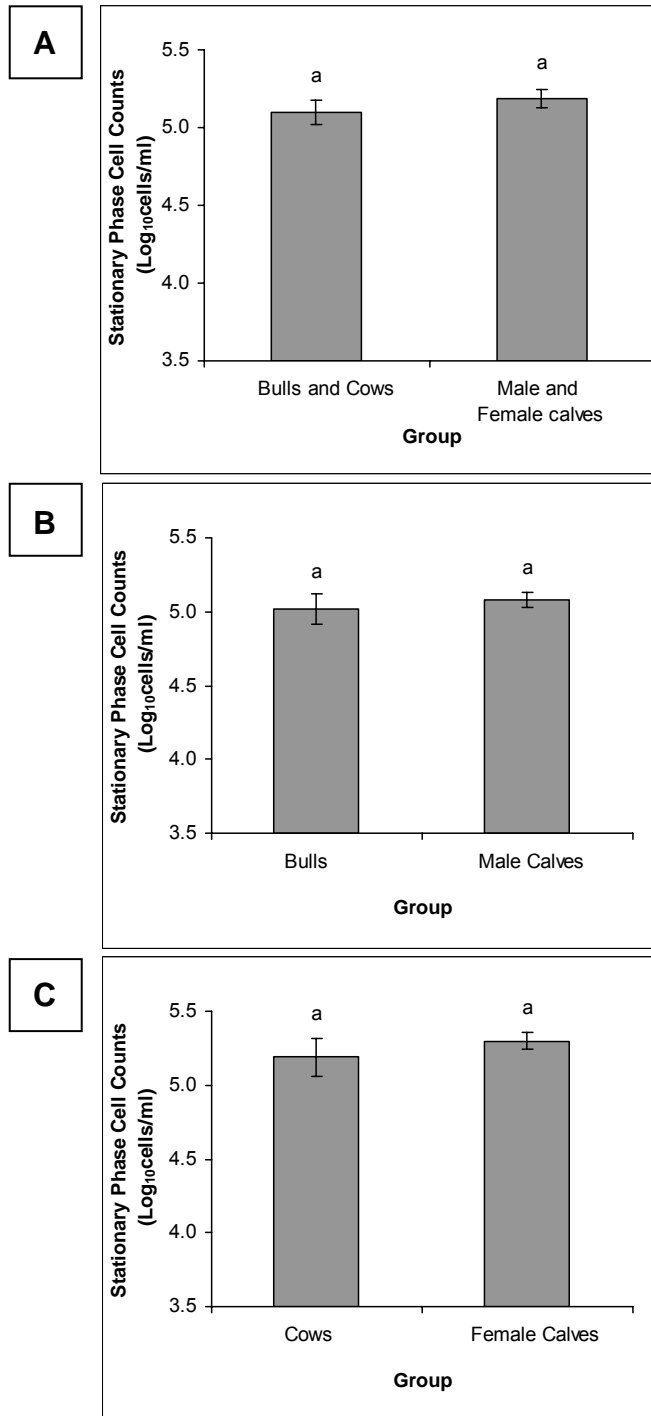


Figure 14. Stationary phase viable cell counts for bovine cell line at passage 4. A: Comparison by Age (older versus younger), Bulls and Cows (n=8) versus Male and Female Calves (n=8). B: Comparison by Age 1 (older males versus younger males), Bulls (n=4) versus Male Calves (n=4). C: Comparison by Age 2 (older females versus younger females), Cows (n=4) versus Female Calves (n=4). <sup>a,b</sup>Different superscripts indicate significant difference (P<0.05).

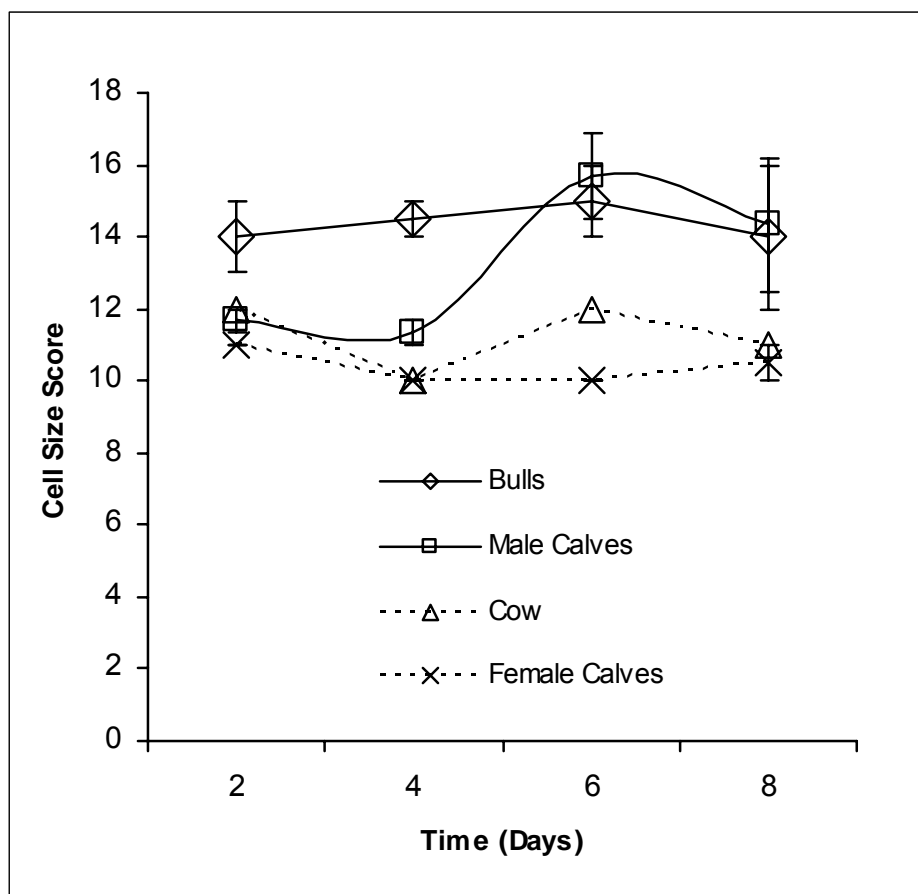


Figure 15. Distribution of sizes of bovine cells from representative animals from which cell lines were derived. Cell size score = sum of the point of each cell (10 cells/cell line). Cell size score per group was measured after passage 4 on days 2, 4, 6 and 8. Ten cells per cell line were scored according to one point for those with widest diameters less than 25  $\mu\text{m}$ , and two points for those greater than 25  $\mu\text{m}$ . Bulls (#2505 and #2507), Cow (#1027), Male Calves (#4090, #4033 and #4044) and Female Calves (#4060 and #4075) were used in this study.

Table 8. Bovine cell size scores\* at passage 4 according to sex

Time (days)	Cell size score	
	Female	Male
2	11.3± 0.33 <sup>a</sup>	12.6± 0.68 <sup>a</sup>
4	10.0± 0.00 <sup>a</sup>	12.6± 0.81 <sup>a</sup>
6	10.7± 0.67 <sup>a</sup>	15.4± 0.75 <sup>b</sup>
8	10.7± 0.34 <sup>a</sup>	14.2± 1.20 <sup>a</sup>
Total	10.7± 0.27 <sup>a</sup>	13.7± 0.68 <sup>b</sup>

Females (Cow and Female Calves, n=3) versus males (Bulls and Male Calves, n=5). \*Cell size score = sum of the point of each cell (10 cells/cell line). Cell size score per group was measured after passage 4 on days 2, 4, 6 and 8. Ten cells per cell line were scored according to one point for those with widest diameters less than 25 µm and two points for those greater than 25 µm. <sup>a,b</sup>Different superscripts in the same row means significantly different (P<0.05).

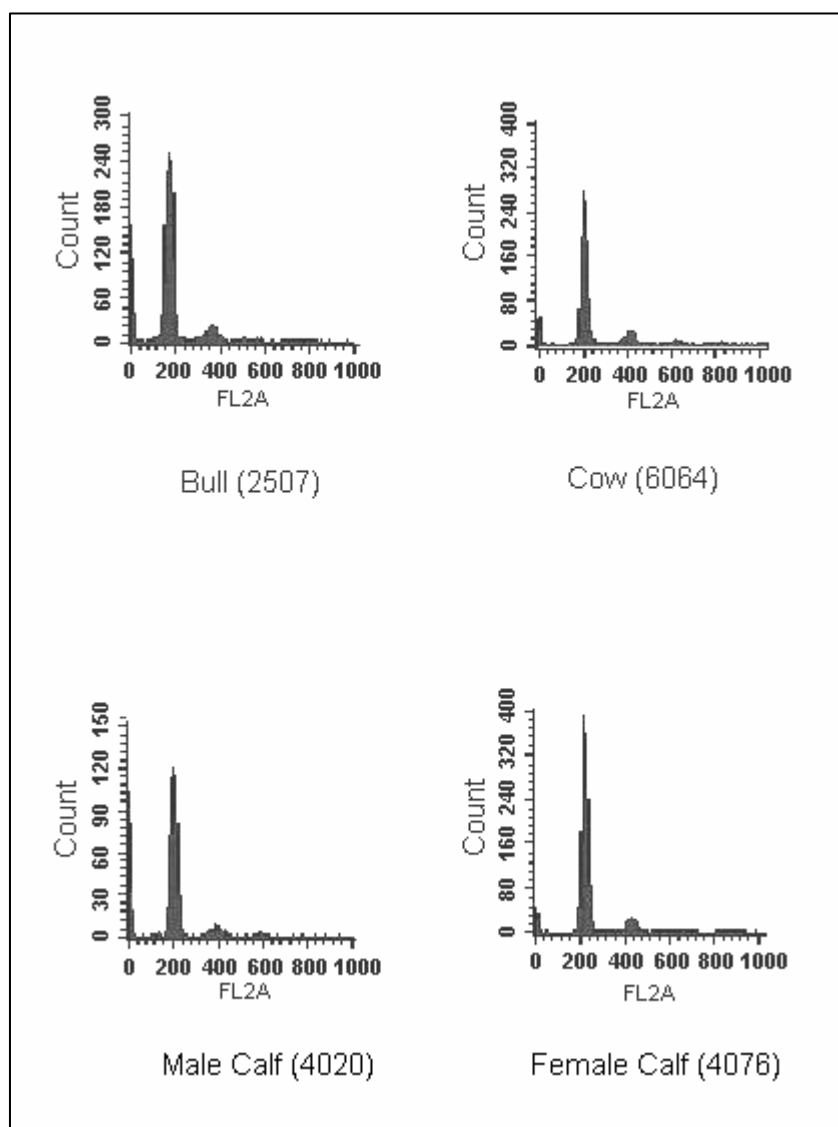


Figure 16. Typical histograms of propidium iodide-stained nuclear DNA from each of the bovine cell line groups. DNA is on the X-axis, where the left most peak represents  $G_0/G_1$  and the smaller peak is  $G_2/M$  phase. The Y-axis is the number of events.

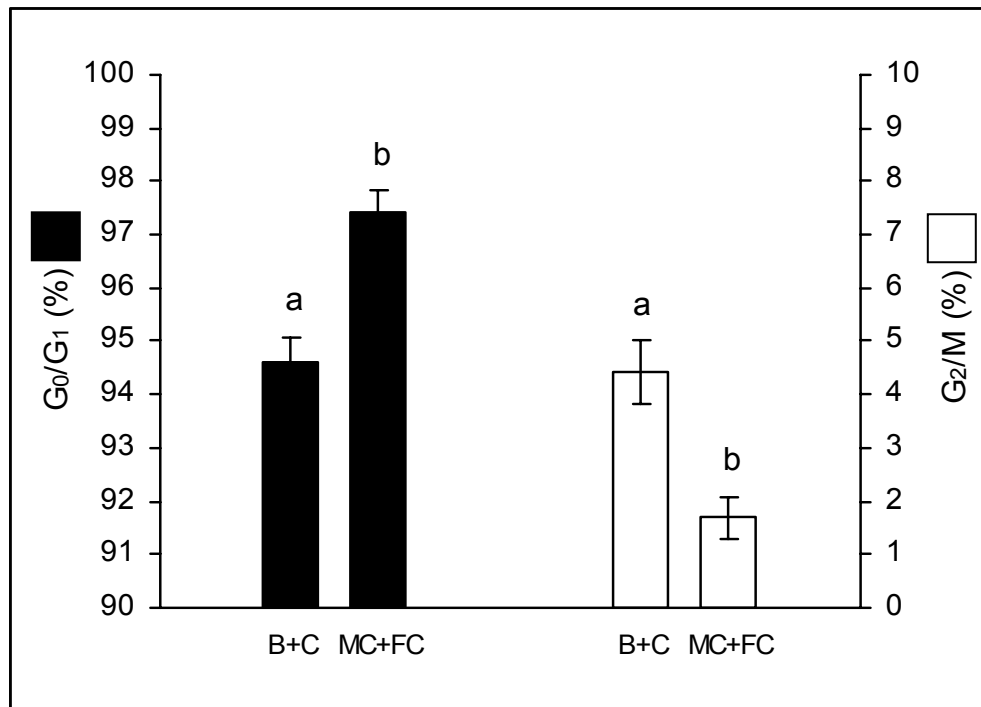


Figure 17. The flowcytometric analysis of bovine cell line nuclear DNA. B+C: Bulls and Cows. MC+FC: Male Calves and Female Calves. <sup>a,b</sup>Different letters on the same type of bars are significantly different ( $P < 0.0005$ ).

Table 9. Comparisons of bovine nuclei in various phases of the cell cycle as analyzed by flow cytometry

Comparisons	Group	Cell cycle phase (mean% $\pm$ SE)			No. of cell lines used
		G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M	
All groups	B	94.76 $\pm$ 0.71 <sup>ab</sup>	0.46 $\pm$ 0.06 <sup>a</sup>	4.78 $\pm$ 0.71 <sup>a</sup>	3
	MC	97.51 $\pm$ 0.43 <sup>b</sup>	0.89 $\pm$ 0.16 <sup>b</sup>	1.60 $\pm$ 0.41 <sup>a</sup>	4
	C	94.48 $\pm$ 0.71 <sup>a</sup>	1.75 $\pm$ 0.45 <sup>ab</sup>	4.16 $\pm$ 0.91 <sup>a</sup>	4
	FC	97.30 $\pm$ 0.91 <sup>b</sup>	0.90 $\pm$ 0.27 <sup>b</sup>	1.80 $\pm$ 0.77 <sup>a</sup>	4
Age	B and C	94.60 $\pm$ 0.49 <sup>a</sup>	1.20 $\pm$ 0.30 <sup>a</sup>	4.43 $\pm$ 0.59 <sup>a</sup>	7
	MC and FC	97.40 $\pm$ 0.45 <sup>b</sup>	0.90 $\pm$ 0.14 <sup>a</sup>	1.70 $\pm$ 0.39 <sup>b</sup>	8
Sex	B and MC	96.33 $\pm$ 0.54 <sup>a</sup>	0.71 $\pm$ 0.12 <sup>a</sup>	2.97 $\pm$ 0.58 <sup>a</sup>	7
	C and FC	95.89 $\pm$ 0.63 <sup>a</sup>	1.32 $\pm$ 0.27 <sup>a</sup>	2.98 $\pm$ 0.62 <sup>a</sup>	8

B: Bull group, MC: Male Calf group, C: Cow group and FC: Female Calf group. The comparison Age means older versus younger cell lines (B and C versus MC and FC). Sex refers to males versus females (B and MC versus C and FC). <sup>a,b</sup>Different superscripts within the same column indicate significant differences (P<0.05).



four groups in the S phase ( $P>0.05$ ) (Figure 17, Table 9). From the pairwise comparisons in conjunction with age, significant differences were noted between older (B and C) groups and younger (MC and FC) groups. C and B groups showed lower percentage of  $G_0/G_1$  phase ( $P=0.0001$ ) and higher percentage of  $G_2/M$  phase ( $P=0.0002$ ) than younger groups (MC and FC) (Table 9).

Growth curves of cell lines showed similar patterns between groups. The Bull group contained one cell line (#1014 bull), which had lower SPCC than others within that group (Figure 18). The C group contained one cell line (cow #580) which had conspicuously lower SPCC than others within the group (Figure 19). As with passage 1 (Figures 9 and 10), the morphology of most cells at passage 4, as shown by microscopy, were fibroblast-like with spindle shapes and many pseudopodial extensions with leading and trailing edges (Meivar-Levy *et al.*, 1997; Lories *et al.*, 2003; You *et al.*, 2004).

When considering familial lineage and MGT (Figure 20), there were no significant relationships between sire and/or dam and their offspring within group and over all lineage groups. Even though both sire and dam had comparatively similar MGT length within group and over all groups, the offspring did not always follow that of sire and dam. In case both bull #110 and cow #1027 had comparatively shorter MGT within group and over all groups, the offspring (female calf #4076) showed comparatively longer MGT. When both bull #1014 and cow #6064 had comparatively longer MGT within group and over all groups, the offspring (male calf #4090) showed comparatively longer MGT. In all but one case, significant differences were noted between cellular growth parameters between the related and unrelated groups ( $P>0.05$ ) (Table 10). However, one difference was found in lag time between #6064 cow related and its unrelated group ( $P<0.05$ ).

Each cell line showed high cell viability ( $95.35 \pm 0.79$ ,  $95.70 \pm 0.72$ ,  $95.71 \pm 0.72$ , and  $96.27 \pm 0.99\%$  for Bulls, Male Calves, Cows and Female Calves, respectively) over all days during the growth curves.

At the fourth passage of the bovine cell lines there were no significant differences in the mean cell generation time (MGT) over all groups (Table 6), but the MGT of each cell line of younger groups (Male Calves and Female Calves) at passage 4 was much shorter than that of passage 2 ( $P<0.05$ ). At passage 4, there was no significant difference in MGT between younger (Male Calves and Female Calves) and older groups (Bulls and Cows) ( $P>0.05$ ) (Table 6). In all pairwise comparisons (Table 7), MGT was not influenced by age or sex.

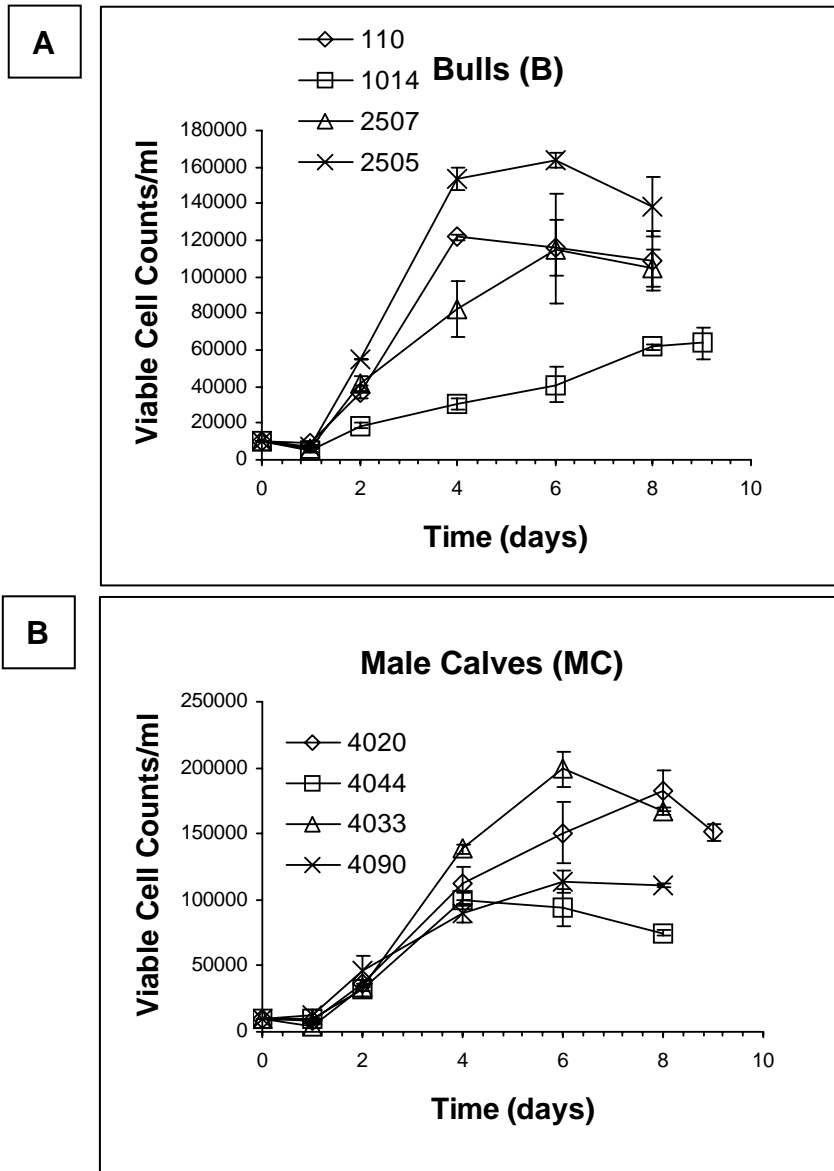


Figure 18. Growth curves of bovine cell lines at passage 4. A: Bull group (#110, #1014, #2505 and #2507). B: Male Calf group (#4020, #4044, #4033 and #4090).

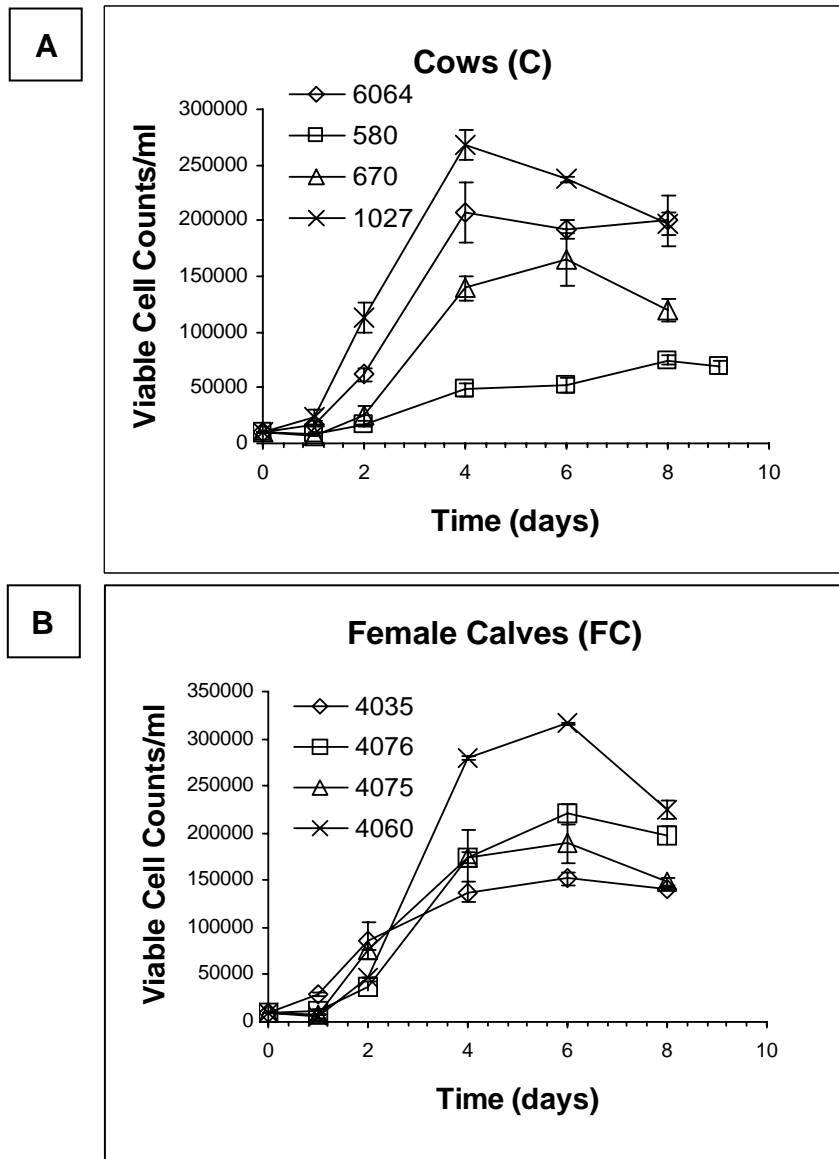


Figure 19. Growth curves of bovine cell lines at passage 4. A: Cow group (# 6064, #580, #670 and #1027). B: Female Calf group (#4035, #4076, #4075 and #4060).

Table 10. Growth pattern comparison of the bovine cell line by familial lineage history at passage 4

Group	No. of animals	Lag time (days)	Stationary Phase cell count (Log <sub>10</sub> cells/ml)	Mean generation Time (days)
Bull #1014 Related	6	0.88±0.21 <sup>a</sup>	5.12±0.10 <sup>a</sup>	0.86±0.12 <sup>a</sup>
Bull #1014 Unrelated	10	0.59±0.17 <sup>a</sup>	5.16±0.06 <sup>a</sup>	0.84±0.05 <sup>a</sup>
Bull #110 Related	4	0.41±0.24 <sup>a</sup>	5.16±0.08 <sup>a</sup>	0.82±0.04 <sup>a</sup>
Bull #110 Unrelated	12	0.80±0.16 <sup>a</sup>	5.14±0.06 <sup>a</sup>	0.85±0.07 <sup>a</sup>
Cow #6064 Related	2	0.00±0.00 <sup>a</sup>	5.16±0.16 <sup>a</sup>	1.06±0.14 <sup>a</sup>
Cow #6064 Unrelated	14	0.80±0.13 <sup>b</sup>	5.14±0.05 <sup>a</sup>	0.81±0.05 <sup>a</sup>
Cow #670 Related	2	1.18±0.24 <sup>a</sup>	5.21±0.05 <sup>a</sup>	0.69±0.16 <sup>a</sup>
Cow #670 Unrelated	14	1.63±0.14 <sup>a</sup>	5.14±0.06 <sup>a</sup>	0.87±0.06 <sup>a</sup>

<sup>a,b</sup> Different superscripts within the same column indicate a significant difference (P<0.05).

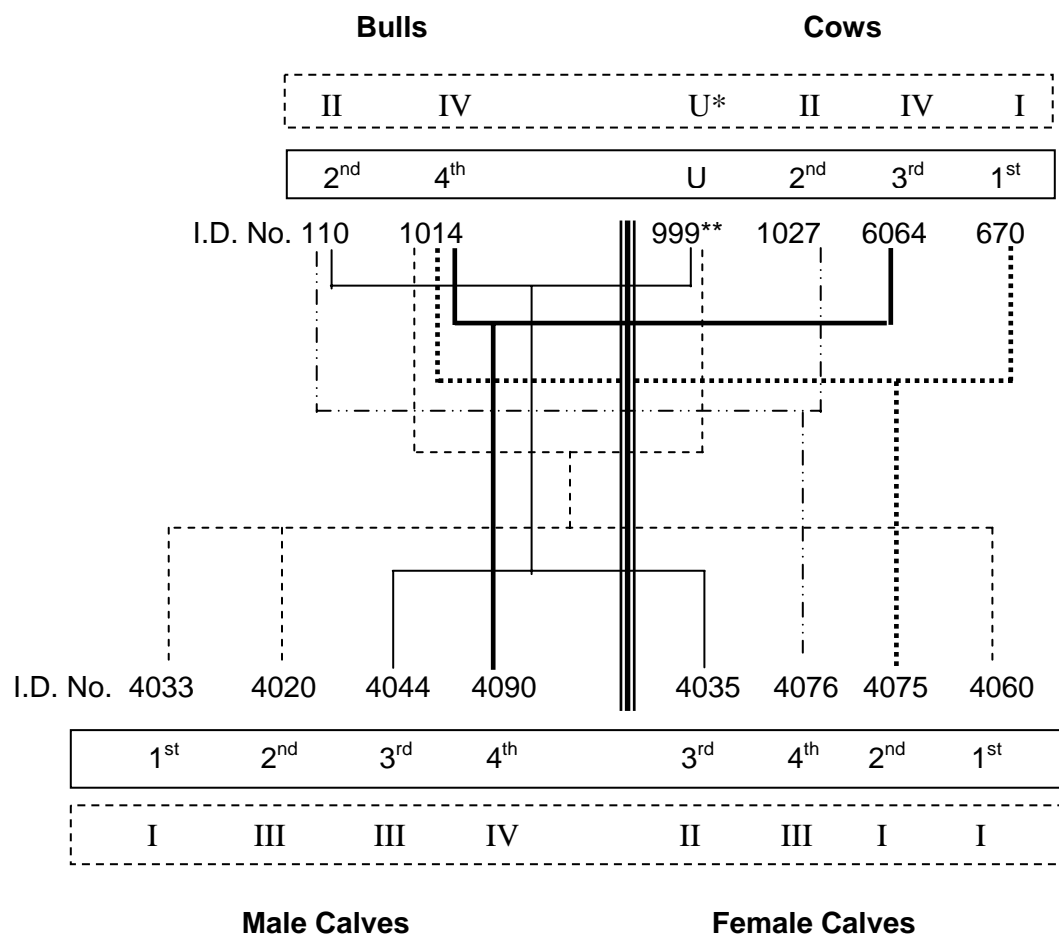


Figure 20. Familial lineage and mean cell generation time cross comparison at passage 4. \*U: Unknown. \*\*Cow #999: No data on these cows were obtained on mean cell generation time. Bull #110 x cow #999 produced male calf #4044 and female calf #4035. Bull #110 x cow #1027 produced female calf #4076. Bull #1014 x cow #999 produced male calves #4033, #4020 and female calf #4060. Bull #1014 x cow #6064 produced male calf #4090. Bull #1014 x cow #670 produced female calf #4075.  : Comparison within group. Each individual was ranked from 1<sup>st</sup> to 4<sup>th</sup> within each group.  : Comparison over all groups. Each individual was ranked from I to IV over all groups: I-1<sup>st</sup> to 4<sup>th</sup>, II- 5<sup>th</sup> to 8<sup>th</sup>, III- 9<sup>th</sup> to 12<sup>th</sup> and IV-13<sup>th</sup> to 16<sup>th</sup>.

Table 11. Ranking of mean cell generation time and lineage relationship of each bovine cell line at passage 4

Group	ID	Age (Yrs)	Mean generation time (days)	Ranking within each group*	Ranking over all groups*	Ranking over all groups
<b>Bulls (B)</b>	110	4.25	0.74	2	6	II
	1014	3.25	1.25	4	16	IV
	2507	1.70	0.81	3	10	III
	2505	1.70	0.73	1	5	II
	Mean±SE	2.73±0.63	0.88±0.12 <sup>a</sup>			
<b>Male Calves (MC)</b>	4020	0.30	0.80	2	9	III
	4044	0.25	0.85	3	11	III
	4033	0.25	0.61	1	2	I
	4090	0.20	1.20	4	14	IV
	Mean±SE	0.25±0.02	0.87±0.12 <sup>a</sup>			
<b>Cows (C)</b>	6064	8.50	0.92	3	13	IV
	580	4.25	1.22	4	15	III
	670	4.25	0.67	1	3	I
	1027	3.30	0.77	2	8	II
	Mean±SE	5.08±1.16	0.89±0.12 <sup>a</sup>			
<b>Female Calves (FC)</b>	4035	0.25	0.76	3	7	II
	4076	0.20	0.91	4	12	III
	4075	0.20	0.70	2	4	I
	4060	0.25	0.57	1	1	I
	Mean±SE	0.23±0.01	0.74±0.07 <sup>a</sup>			

\*Each individual was ranked from 1<sup>st</sup> to 4<sup>th</sup> within each group, or ranked from I to IV over all groups: I-1<sup>st</sup> to 4<sup>th</sup>, II-5<sup>th</sup> to 8<sup>th</sup>, III- 9<sup>th</sup> to 12<sup>th</sup> and IV- 13<sup>th</sup> to 16<sup>th</sup>.

<sup>a,b</sup>Different superscripts within the same column indicate a significant difference (P<0.05).

Therefore, the age effect on the MGT of each cell line was not apparent at passage 4. In this culture system, the MGT of each cell line was influenced by age-related factors at passage 2, however, by passage 4 the MGT was not influenced by either sex or age.

Over all groups, cell lines showed lag times with the average  $0.70 \pm 0.13$  days and a coefficient of variance was 76.86% (Table 6). When compared with the CV of 80.32% at passage 2 (Table 2), the scattered pattern of each cell line lag time over all groups was similar in passage 4. Numerically the average lag time over all groups was decreased from  $1.27 \pm 0.26$  (passage 2) to  $0.70 \pm 0.13$  days (passage 4). However, there was no statistical difference ( $P > 0.05$ ). The lag time of female groups (Cows and Female Calves) at passage 2 were significantly decreased compared with that of female groups (Cows and Female Calves) at passage 4 ( $P < 0.05$ ). In other pairwise comparisons considering lag time at passage 4, there were no significant differences among groups (Table 7). In all group and pairwise comparisons, lag time was not to be influenced by age or sex.

That there was a dramatic decrease in average lag time in female groups (Cows and Female Calves) from passage 2 to passage 4 is a topic for further investigation. Mammalian cell growth is impacted by growth factors (Gospodarowicz *et al.*, 1974; Adamson, 1990; Butt *et al.*, 2003) and inhibitors (Slingerland *et al.*, 1994), and specific gene expression levels involved in logarithmic growth is an active area of research.

There were no significant differences in stationary phase viable cell counts (SPCC) between groups (Table 6). However, the SPCC of the Female Calves group was numerically the highest, followed by Cows, Male Calves, and Bulls groups (Table 6). With regard to the sex of the donor animal and SPCC, in most comparisons, the male SPCC was significantly lower than that of the female ( $P < 0.05$ ) (Figure 12 and 13). In comparing Bulls and Cows groups versus Male Calves and Female Calves, Male Calves versus Female Calves, and Bulls versus Female Calves, significant differences were noted in SPCC ( $P < 0.05$ ). The SPCC results of passage 2 were different from those of passage 4 in that SPCC of younger groups (Male Calves and Female Calves) was higher than that of older groups (Bulls and Cows) ( $P < 0.05$ ). As this pattern was not maintained through passage 4, age-related factors did not impact SPCC, however, sex-related factors were more prominent in SPCC.

Throughout most of the growth curve at passage 4 (Figure 15) (Table 8), female cell lines were smaller compared with male cell lines. Sex-related factors may influence the selective generation of small-sized cells or the maintenance of small-sized cells during growth of female cell lines, thereby being reflected by high SPCC.

## Discussion

In the first production of a mammalian offspring from adult somatic cells, most of the donor cells were arrested in a quiescent stage ( $G_0$ ) by serum starvation (Wilmut *et al.*, 1997). In reports of viable embryos from NT (Campbell *et al.*, 1996; Collas *et al.*, 1992; Prather, 1996), usually better efficiencies have been obtained with the use of  $G_0/G_1$  phase than other cell phases (Campbell *et al.*, 1996; Collas *et al.*, 1992; Prather, 1996). Thereby, the use of  $G_0/G_1$  nuclei for NT can be regarded as a preferred technology. Consequently, the age of the donor animal should be a factor to consider in choosing cell lines for NT, especially when  $G_0/G_1$  nuclei are intended for use as karyoplasts.

In case of primary Schwann rat cells, similar growth rates between large- and small-sized cells were noted in DMEM with 3% fetal calf serum (Conlon *et al.*, 2003). In yeast, cell size check point was accepted as a marker of cell division and cell growth (Hartwell *et al.*, 1974). Thus far, however, checkpoints in mammalian cells have not been established (Conlon *et al.*, 2001; Grewal and Edgar, 2003; Weitzman, 2003). If it was the case in mammalian cells, there will be a possibility that, in female bovine cell lines, more cells could be arranged in limited area than those of male cell line. With similar growth rates between small- (less than 25  $\mu\text{m}$ ) and large-sized cells, the cell population dominated by small-sized cells could establish more cells in limited area during 8 days of *in vitro* culture.

Cell cycle analysis at passage 4 on day 0 of culture showed that over 90% of cells were in  $G_0/G_1$  portion in each cell line of all groups (Table 9). When analyzed by age, age of donor animal was influential on the percentage of cells in  $G_0/G_1$  on day 0 at passage 4. Cell lines from younger donors were more frequently at higher  $G_0/G_1$  percentages or synchronized than those derived from older donors.

There are some alternative methods other than serum starvation to synchronize cells including contact inhibition or reversible cycle inhibitors such as roscovitine, aphidicolin, and butyrolactone 1 (Wang, 1991; Levenson and Hamlin, 1993; Kitagawa *et al.*, 1994; Boquest *et al.*, 1999; Kues *et al.*, 2000; Gibbons *et al.*, 2001).

In feline mammalian cell lines, Gómez *et al.* (2003) reported that 84% or higher synchronization into  $G_0/G_1$  phase synchronization was achieved in domestic cat fibroblast cells by serum starvation, contact inhibition and roscovitine treatment (96, 88, and 84 %, respectively). In porcine fetal fibroblasts, short times in serum deprivation (24 to 72 hours) produced 77.9 to 80.2% of cells in the  $G_0/G_1$  phase (Kues *et al.*, 2000). When compared with



these reports, the results in experiment show comparatively high synchronization status on day 0 by contact inhibition.

When comparing familial lineage (Figure 2) (Table 10), most comparisons showed no significant differences in lag time, SPCC and MGT between related and unrelated groups. Because of the single difference noted in lag time between cow #6064 related and the unrelated group, further investigations into lag time between such groups may be needed.

In familial lineage analysis of MGT (Figure 20) (Table 11), there was no evidence that the phenotype of sires and dams was influential to offspring in terms of MGT. Furthermore, most rankings (within group or over all groups) conferred to each cell line at passage 2 (Figure 11) (Table 5) were changed by passage 4 (Figure 20) (Table 11), whereby, there were marked changes of comparative MGT within group or among all groups, with 8 cases of 15 (60%) and 5 cases of 15 cell lines (33%), respectively. It may be that the inherent genetic controls over MGT had not been effective in earlier passages (until passage 4), and that subsequently the *in vitro* environment affected the MGT of each cell line.

Mueller *et al.* (1980) reported that cellular senescence in a cloned strain of bovine fetal aortic endothelial cells had a replicative lifespan of 80 cumulative population doublings. In Clark *et al.*'s report (2003), after a 60 days of *in vitro* culture, ovine cell lines started to show MGT delay, which led to cell senescence (Clark *et al.*, 2003). If this is the case, it may be, that specific genes for telomere resistance controls were not effective in early passages (until passage 4). If the telomere controlling genes (i.e., genes encoding telomeric repeat binding factor [TRF] and/or telomere reverse transcriptase [TERT]) (Moyzis *et al.*, 1988; Hastie *et al.*, 1990; Harley *et al.*, 1990; Karlseder *et al.*, 2003) were related to MGT and eventually modified proliferative lifespan of each mammalian cell line thus, the telomere resistant genes could be effective at later passages (> 60 days after *in vitro* culture). Because at early passages, not much difference in telomere length is expected even though telomere resistant gene effects were expressing during cell proliferation.

In addition to the specific gene effects related to telomere resistant, there is another possible explanation that bovine cell lines at passage 2 were susceptible to age factor, as evidenced by MGT. That factor could be from *in vivo* characteristics of each cell line. However, at passage 4, through consecutive exposure to *in vitro* conditions, cell lines were prone to be adjusted in uniform status into similar MGT. Nichols *et al.* (1977) found that human diploid fibroblast-like cell strain IMR-90-derived from one female embryo showed three different doubling capacities according to conditions including seeding density, growth

medium, medium volume and subcultivation reagents. Smith and Whitney (1980) reported that even the two human fibroblast cells derived from a single mitosis showed different population doubling capacity. You *et al.* (2004) established extended lifespan (delayed senescent passage) bovine fibroblast cells from primary bovine embryonic fibroblasts by optimizing culture conditions. Our study also showed some extrinsic factors to consider. By the extrinsic changes of each cell line at passage 4 from *in vivo* to *in vitro* (serum, temperature, air, humidity), from frozen to thawing (biological and mechanical change from cryopreservation process) (Rall and Fahy, 1985; Vajta *et al.*, 1998), and from heterogeneous cells to homogenous fibroblasts (surface attachment natural selection along with passage progression) (Zimmermann *et al.*, 2001), the growth pattern of each cell line could have changed. Though this was only included data on passage 2 and passage 4, it gives motivation to do further research in this field.

Each cell line also showed constant viability (average through the growth curve patterns 94.36 to 97.98%) at passage 4. However, compared with that of passage 2, all groups showed decreased cell viability during the growth curve at passage 4 ( $P < 0.05$ ). Though, numerically over 90% of cell viability is not a comparably low viability after freeze-thawing processes when compared with that of previous reports (Lovelock and Bishop, 1959; Kelbe and Mancuso, 1983; Rall and Fahy, 1985; Reubinoff *et al.*, 2001; Corsini *et al.*, 2004; Pascual *et al.*, 2004), the influence of the cryopreservation process on the viability of bovine cell line can not be ignored.

In conclusion, it is clear that the MGT of each cell line was influenced by age at passage 2, however, by passage 4 the MGT of each cell line was not affected by either sex or age. Thus, as passages continue, other extrinsic environmental factors could have influenced the MGT of each bovine cell line. The adjustment of culture conditions, which are known to be able to influence MGT and/or population capacity would be very important research related to NT in future studies. At passage 4, in most comparisons related to sex, the male cell lines showed lower SPCC than female groups ( $P < 0.05$ ). In case of female cell lines, sex-related factors can give influence either on the generation of small-sized cells or small-size cell maintenance during growth curve with female cell lines. In the present study, by contact inhibition, cell lines from younger donors were more frequently at higher  $G_0/G_1$  percentages or synchronized than those derived from older donors. Therefore, age of donor animals should be considered as a factor in choosing cell lines for NT, especially when  $G_0/G_1$  nuclei are intended for use in the process.

## CHAPTER V

### SUMMARY AND CONCLUSIONS

In this study, the influence of sex and age of cattle cell donor during *in vitro* cell culture was evaluated. The hypothesis of this study was that either sex or age of donor animals from which somatic cells were derived influences cell growth pattern *in vitro*. The objective of this study was to investigate the growth patterns of bovine (*Bos taurus*) cells at passage 2 and 4 of *in vitro* growth of cell lines derived from animals of various ages and sexes. Such information can provide a foundation that could be useful in selecting donor somatic cells for nuclear transfer (NT).

Skin biopsies were taken from each of 16 individuals including four Bulls, four Cows, four Male Calves, and four Female Calves. At passage 2, Bulls and Cows have significantly longer mean cell generation time (MGT) than those of Male Calves and Female Calves. Our results indicated that at passage 2 *in vitro* culture of bovine cell line, cell generation time is influenced not by sex as once thought but by age of donor animal.

When evaluating familial lineage, comparison between related and unrelated groups showed that most comparisons do not show significant differences in lag time, stationary phase viable cell counts (SPCC) and MGT.

In the present study, each cell line showed a high level of cell viability throughout the development of cell growth curves (98 to 99%), which indicates that stable cell maintenance and proper cell harvest were conducted prior to and during this study.

Results from the present study showed a significant difference at passage 4 compared with passage 2 (age factor could influence the MGT as group level), indicating that neither age nor sex would likely influence the MGT of groups at passage 4. It was clear that the MGT of each cell line was not influenced by sex but by age at passage 2, however, by passage 4, the age effect on the MGT of each was no longer detected.

Furthermore, with regard to the sex of the donor animal and SPCC, in most comparisons the male SPCC was significantly lower than that of the female ( $P < 0.05$ ). The female bovine cell lines were prone to maintain small-size cells suggesting that in the same surface area more cells could be arranged compared with that of the male cell lines.

Cell cycle analysis at passage 4 on day 0 of this study showed that > 90% of cells were in  $G_0/G_1$  portion in each cell line of all groups. Age of the donor influenced the

percentage of cells in  $G_0/G_1$  on day 0 at passage 4. Cell lines from younger donors were more frequently at higher  $G_0/G_1$  percentages, or synchronized than those derived from older donors. Thus, age of donor animal could be a factor in selecting cell line for NT, especially when  $G_0/G_1$  phase cells are intended for use.

Most comparisons in this study showed no significant differences in lag time, SPCC and MGT between related and unrelated familial lineage groups. Furthermore, there was no significant difference in cell viability all through the growth curves among age and sex groups. Each cell line also showed constant viability (average through growth curve 94.36 to 97.98%) at passage 4.

In conclusion, in this study it is clear that the MGT of each cell line was influenced not by sex but by age at passage 2. By passage 4, the MGT of each cell line was not affected by either sex or age. As passages continue, the extrinsic environmental factors (e.g., freezing) likely influenced the MGT. For future studies, the adjustment of culture conditions which are known to alter MGT and/or population capacity would be very important for further research. In contrast, in most comparisons by sex, female groups showed higher SPCC than male groups. A sex-related factor was found to influence the generation of small-size cells and small-size cell maintenance during growth curves with female cell lines. In the present study, cell lines from younger donors were more frequently at higher  $G_0/G_1$  percentages, or synchronized than those derived from older donors by contact inhibition. We conclude that age of donor animal should be considered as a factor in selecting early passage cell lines for NT.

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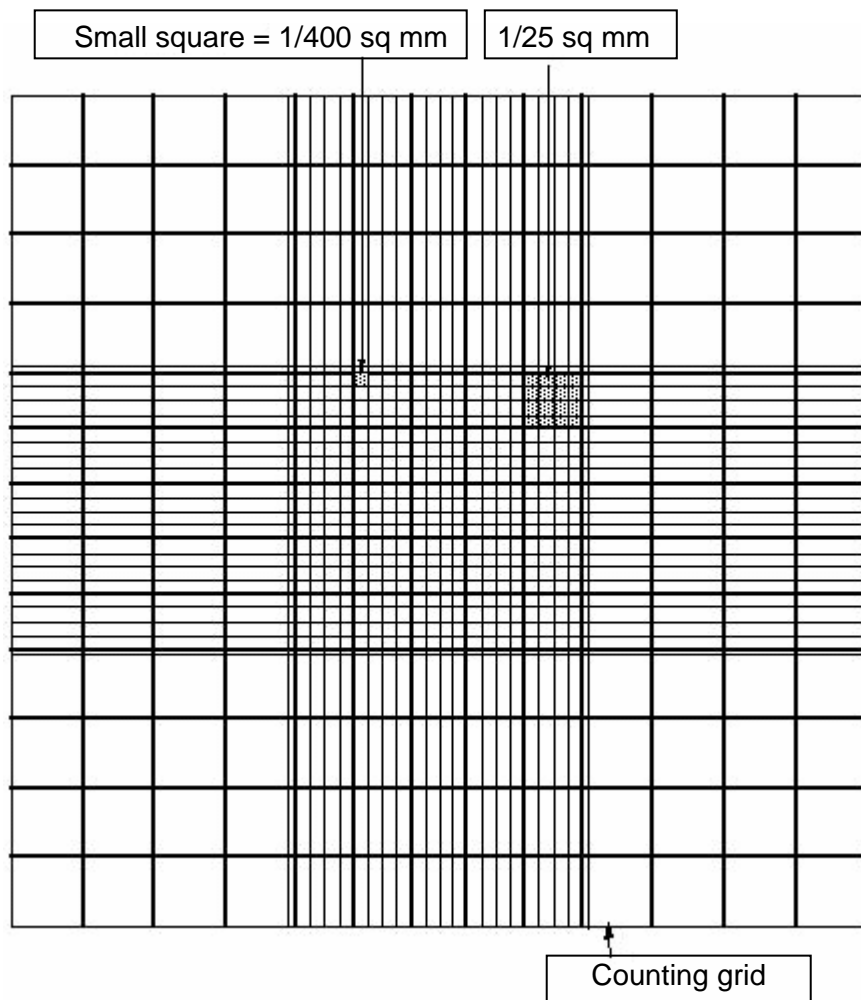
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**APPENDIX**  
**THE COUNTING GRID STRUCTURE OF THE HEMOCYTOMETER**



## **VITA**

Jeho Shin was born in Seoul, Korea, on January 10, 1969, and raised by his parents Kyung-soon Lee and In-sik Shin. After graduation from Jayang High School in Seoul, Korea, he attended KonKuk University in Seoul and received his Bachelor of Science degree in dairy science in 1991.

He enrolled at Kon Kuk University in Seoul to pursue a graduate degree in dairy science. Under the direction of Professor Jae-Hyeun Yu, Jeho earned a Master of Science degree in dairy science in the summer of 1994. He has worked as researcher at Korea Yakult Research and Development Center until 2000 doing lactic acid bacteria and beverage development research. He enrolled at Louisiana State University to pursue a graduate degree in animal sciences. He is now a candidate for the degree of Master of Science in the interdepartmental program in animal and dairy sciences at Louisiana State University, Baton Rouge, Louisiana, under the supervision of Professor Robert A. Godke.